IMPROVING THE YIELD AND QUALITY OF BLACKCURRANT (*Ribes nigrum* L.) EXTRACTS

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DECLARATIONS

This thesis does not contain any material, which has been accepted, for a degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge, this thesis contains no material previously published or written by another person except where due acknowledgement is made

Sandra Marlan Garland

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This thesis is dedicated to Michael Thomas Garland

'Spring is sprung
The grass is ris'
I wonder where the birdies is
Some say the birds are on the wing, but that's absurd
The wings are on the bird'

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ABSTRACT

Aspects of harvest, post-harvest storage and extraction technology were investigated with view to improving yield and quality of blackcurrant bud extracts. Sieving extraneous material from machine harvested buds increased yield by 20 % consuming 8 % less solvent. New extraction technology in which buds were steeped in ethanol prior to partitioning against petroleum ether increased the yield of volatiles by 29 % whilst reduced the yield of acids by 61 %. The extracts had a more pungent catty aroma reminiscent of the European product. The chemical profile of extracts from new clonal material selected from cv. White Bud was found to contain higher levels of 4-methoxy-2-methyl-2-butanethiol. This component confers a 'catty' note associated with quality extracts. The new selection differed from White Bud by having higher levels of sabinene, myrcene, bicyclogermacrene and hardwickic acid.

Studies focused on the levels of the endogenous thiol which was depleted by 85 % in extracts stored for less than a month. Freezing of buds also resulted in a loss of 50 % of thiol and 18 % of other volatiles after 24 hours. Research on harvest technology showed that cutting buds by hand increased thiol concentration (4.6 $mgkg^{-1}$ DMB) compared to those extracted from machine-harvested (3.5 $mgkg^{-1}$ DMB). Post-harvest thiol production was most rapid in fresh hand-cut buds. Mechanical harvesting resulted in a loss of thiols and reduced post-harvest production in fresh buds. Volatile concentrations were reduced when machine-harvested buds were further damaged by rolling.

Damage to bud structure by rolling resulted in depletions of mono and sesquiterpenes and had a significantly greater detrimental effect in the retardation of post-harvest production than did freezing. Rolling did not stop synthesis of oxygenated sesquiterpenes and diterpene acids whilst freezing deactivated these processes. Disparities in the behaviour amongst terpenoid families were consistent with sub-cellular compartmentalisation.

The dry weight of buds and yield of volatiles decreased throughout dormancy. Diterpene acids increased as bud burst approached. Commercial scale-incubations for

72 hours produced a 29 % increase in the level of 4-methoxy-2-methyl-2-butanethiol and an 8 % decrease in oil yield. A novel synthetic method using methyl-3-methoxypropionate to produce a butanol intermediate with thiolation using Lawesson's reagent was developed. A non-volatile cysteine thiol conjugate thought to be the precursor to volatile thiols was successfully detected in blackcurrant. Levels of the conjugate and the thiol in the new clonal buds were 3.3 and 4.6 fold higher, respectively, than that detected in the White Bud. An inverse relationship in the levels of the thiol and those of the conjugate during the final stages of dormancy up until bud burst was elucidated.

ABBREVIATIONS

BHA	-	butylated hydroxy anisole
CE	-	collision energy
DMB	-	dry matter basis
FID	-	flame ionization detector
FPD	-	flame photometric detector
HPLC	-	high pressure liquid chromatography
HTC	-	high thiol clones
GC	-	gas chromatography
LR	-	Lawesson's reagent
MFT	-	2-methyl-3-furanthiol
MS	-	mass spectrometry
MS/MS	-	daughter/daughter fragmented mass spectrometry
MSD	-	mass selective detector
RVE	-	rotary vacuum evaporation
SD	-	standard deviation

INTRODUCTION

The blackcurrant oil industry, as opposed to that of the blackcurrant berry, emerged in Tasmania in the 1980s. The impetus was originally to utilise the prunings from the berry industry but developed into a crop in it's own right. The cut canes are stripped of their buds immediately prior to bud burst in late winter and the oil from the buds is extracted to produce a viscous green/yellow oleoresin also called 'concretes'. The pungent oil is used in perfumes, confectionary and to strengthen the aroma impact of blackcurrant berry products. The production of blackcurrant oil in Europe has been well established for many years and the French extract has been prized for its pungency and complexity of aroma. Despite the successful introduction of the Tasmanian product, internationally markets regard the European oils as superior. This lack of competitiveness must be addressed predominantly by tailoring the Tasmanian product to meet market specifications in terms of aromatic profile and consistency.

Blackcurrant oils are a complex mixture and the contribution of each component to the aroma impact varies widely depending on odour thresholds and concentration. One of the most important compounds, and one that is found in high concentrations in the French product, is the naturally occurring thiol, 4-methoxy-2-methyl-2-butanethiol which contributes what is described as a 'catty' note. Increasing the level of this thiol and other volatile components associated with high quality oils would be a significant step towards improving the competitiveness of the blackcurrant oil industry in Tasmania!

The aim of this project is to conduct an empirical investigation to establish the conditions of harvest, post-harvest and extraction which impact on the yield and quality of blackcurrant extracts and elucidate the chemistry underlying the biosynthesis of the endogenous thiol which confers the 'catty' note characteristic of the family, Ribes nigrum L.

Funded by industry, in conjunction with government sponsership, the directive of this study was to achieve improvements in the yield and quality of blackcurrant extracts, preferably within the constraints of established infrastructure. What then are the

aspects fundamental to blackcurrant oil yields and quality? Obviously the variety, age, nutrition and plant management contribute to blackcurrant oil composition. Research at the University of Tasmania into nutritional requirements, spacing and planting density, harvest methods and extraction technology contributed to the successful development of a viable industry (Kerslake, 1984; Kerlake and Menary, 1985; Kerlake, 1986; Kerlake and Le Quéré, 1989; Menary, 1986, Menary, 1990; Poulter, 1991). However, aspects of the later stages of oil production in the areas of harvest, storage of buds and in the extraction process itself were identified by industry as areas in which research could be advantageous. The terms of reference in this study then is the investigation of the fundamental aspects that contribute to blackcurrant extract yield and quality from the time of leaf abscission, through the dormant stage of buds and up until bud burst. As a directed study the primary focus will be to investigate processes that industry can adjust and optimise in the areas of harvest, storage and the extraction procedures to optimize quality and yield of extract.

How then can we maximise the yield and quality from buds within the framework of bud dormancy, bud burst, harvesting extraction and storage. In Tasmania, Australia, the main commercial blackcurrant cultivar is White Bud, which is a selection from the widely-grown European cv. Baldwin. Higher levels of 4-methoxy-2-methyl-2-butanethiol have been detected in some plants of cv. White Bud grown at Bushy Park in Southern Tasmania (pers. com. R.C. Menary). The increased level of 4-methoxy-2-methyl-2-butanethiol in the clonal material propagated at the University of Tasmania may be accompanied by characteristic variations in the levels of other volatiles. Elucidation of the chemical profile may establish the relationships between the high thiol containing selections, White Bud and other favourable varieties.

The coordination and timing of the harvesting of buds in Tasmania from July to late August is limited by the availability of the mechanical harvester (pers. comm. Essential Oils of Tasmania Pty. Ltd.) however, there appears to be some scope to adjust harvest time to maximise the desired oil composition and yield. Manipulation of harvest time has the potential to significantly alter the quality and yield of blackcurrant extract.

Mechanised bud harvesting is now standard in Tasmania. Prior to the development of the machine harvester, buds were cut from the cane by hand and it has been shown that the extracts from hand-cut buds are of higher quality (Menary, 1986). The damage to bud structure by machine-harvesting may initiate metabolic, catabolic and oxidative alteration of oil composition. Although the non-viability of the traditional hand-cut methods entrenches the continued use of the machine-harvester, improved yield and quality may be obtained by removal of miscellaneous material from harvested buds. In addition identifying which factors contribute most to the alteration in the chemical profile of oils may present practical solutions to minimize detrimental procedures. In fact, terpenoid accumulation often occurs as non-volatile consitituents such as terpene diphosphates and flavorless glycosides (Croteau, 1987; Winterhalter and Skouroumounis, 1997). These many aromatic volatile compounds bound to glycosides have been shown to be released during storage (Crouzet and Chassagne, 1999). The activation of enzymatic and oxidative processes has the potential to improve or detract from the quality of extracts from plant material by releasing aglycones or oxidising and metabolising existing terpenoids. The manipulation of harvest and storage conditions, then, may present many opportunities to alter the extract composition of blackcurrant buds.

Fundamental to extract quality is the process of extraction itself. Many aspects of extraction technology are limited by the infrastructure and practicalities of industrial processes. Indeed the types of solvent suitable are restricted to those which are non-toxic or carcinogenic, that are sufficiently volatile to be removed from the final product and do not impart un-favorable notes to the aroma profile. None the less extraction conditions and procedures provide scope to improve yield and quality.

The empirical approach to research in industrial based processes often has the advantage of direct application. However the importance of the thiol, 4-methoxy-2-methyl-2-butanethiol, to the impact of blackcurrant extracts cannot be underestimated and the behaviour and chemistry of the biosynthesis and depletion of this chemical receive considerable attention in this study. Elucidating the chemistry of thiol production in blackcurrants would provide for the adjustment of processes to

maximize conditions favourable to the production and maintenance of this important component.

LITERATURE REVIEW

Blackcurrant (Ribes sp.) (family: Grossulariaceae, order: Rosales) is cultivated in temperate and cold climates and the small black smooth currants are harvested to produce juices, jams and liquours. In the early 20th century four distinct varieties; Baldwin, Boskoop Giant, French and Goliath, were identified (Hatton, 1920). The authoritative key to identifying well-established blackcurrant varieties has been published (Todd, 1962). Of the numerous clonal selections that have since eventuated, the predominant cultivar used in commercial operations in Tasmania, Australia is White Bud, a selection from the Baldwin variety imported from the United Kingdom. It is considered to be less susceptible to frost damage and has a growth pattern favourable to the penetration of sunlight.

When the leaves are crushed the characteristic acrid impact associated with the plant is released. Being a deciduous perennial, an oil, that captures the aroma impact, is sequestered during summer into the developing leaf and fruit bud. The buds remain dormant through winter following leaf senescence and abscission. The oil is extracted from blackcurrant dormant bud prior to bud burst. Commercial operations now cut the canes in mid winter when the plants are dormant, ensuring sufficient basal buds are left to produce new shoots in spring. The buds are stripped from the cut canes and extracted with low polarity organic solvents to produce a viscous green/yellow oleoresin also called 'concretes'. Alcoholic extracts from the oleoresins are known as absolutes. Both blackcurrant absolutes and concretes are used in perfumes, cosmetics and to fortify the essence of blackcurrant in fruit products.

THE TASMANIAN INDUSTRY

On the island state of Tasmania located south east of mainland Australia the temperate climate provides conditions suitable for the cultivation of blackcurrant. Although the State had a small blackcurrant fruit industry it wasn't until the 1980's that the infrastructure for the production of blackcurrant oil was established. Research into nutritional requirements, spacing and planting density, harvest methods and extraction technology contributed to the successful development of a viable industry (Kerslake, 1984) with 15 hectares established by 2001. Despite the acceptance of the Tasmanian

product by the international essential oils market the preference for the blackcurrant concrete produced in Europe denied the Australian industry a competitive advantage. Industry required improvements in yield and quality of the blackcurrant extracts to meet this demand. The directive of this study, funded by industry in conjunction with government funding, was to achieve these improvements, preferably within the constraints of established infrastructure.

What then are the aspects fundamental to blackcurrant oil yields and quality? Obviously the variety, age, nutrition and plant management contribute to blackcurrant oil composition. Blackcurrant plants are adaptable to a wide range of soil types though requires good drainage and prefers soils that have a neutral pH.

However, industry was interested in optimising the later stages of oil production in the areas of harvest, storage of buds and bud extracts and in the extraction process itself. The terms of reference then in this study is the investigation of the fundamental aspects that contribute to blackcurrant extract yield and quality from the time of leaf abscission, through the dormant stage of buds and up until bud burst. As a directed study the processes that industry can adjust and optimise in the areas of harvest, storage and the extraction procedures employed will be the primary focus.

Assuming then that the nutritional aspects of the crop management have been managed competently, the maximising of planting with reference to light penetration and accessibility by mechanical harvesters addressed (Kerslake, 1984) and the influence of the age of the plant and the circumstances of the growers considered, how then can we maximise the yield and quality from buds within the framework of bud dormancy, bud burst, harvesting extraction and storage.

BLACKCURRANT BUD COMPOSITION

Research into the components of extracts of blackcurrant bud began following the first product release early last century by the fragrance company, Grasse. A study was conducted by the manufactures using classical identification methods of derivatisation and measurement of physical parameters such as acidity, refractive index etc. Over the century the identification of components proceeded at rates equivalent to the

development of analytical technology such as liquid and gas chromatography and mass spectrometry. The collation of identified chemicals has been undertaken (Andersson *et al.*, 1963; Kerslake, 1984). Table 1 lists the chemicals identified in blackcurrant bud extracts and the researchers who first identified them.

hydrocarbons			
toluene	Latrasse and Demaizieres, 1971		
camphene	Latrasse and Demaizieres, 1971		
Δ_3-carene	Latrasse, 1969, Fridman, 1971		
m-cymene	Le Quéré and Latrasse, 1990		
p-cymene	Schimmel, 1907;Latrasse , 1969; Williams 1972		
δ-cymene	Latrasse and Demaizieres, 1971		
m-cymene	Le Quéré and Latrasse, 1990		
limonene	Fridman, 1971; Latrasse, 1969; Latrasse and Demaizieres, 1971		
β-myrcene	Latrasse, 1969; Latrasse and Demaizieres, 1971		
α-phellandrene	Latrasse, 1969; Latrasse and Demaizieres, 1971; Williams, 1972		
β-phellandrene	Latrasse, 1969; Latrasse and Demaizieres, 1971; Williams, 1972		
α-pinene	Latrasse, 1969; Latrasse and Demaizieres, 1971; Williams, 1972		
β-pinene	Glichitich and Igolen, 1937; Fridman, 1971; Williams, 1972		
sabinene	Glichitich and Igolen, 1937; Williams, 1972		
α-cadinene	Glichitich and Igolen, 1937; Williams, 1972		
β-caryophyllene	Glichitich and Igolen, 1937; Fridmam, 1971; Latrasse, 1969		
α-methylbutene	Williams, 1972		
6,10,14-trimethyl-2-pentadecane	Le Quéré and Latrasse, 1990		
cyclohexane	Williams, 1972		
caryophyllene oxide	Le Quéré and Latrasse, 1990		
(Z)-ocimene	Le Quéré and Latrasse, 1990		
(E)-ocimene	Le Quéré and Latrasse, 1990		
p-methylisopropenyl benzene	Williams, 1972	naphthalene	Kerslake, 1984
cis-ocimene	Williams, 1972	nonan-2-one	Kerslake, 1984
benzene	Williams, 1972	allo-aromadendrene	Kerslake, 1984
ethylbenzene	Williams, 1972	germacrene D	Kerslake, 1984
α-terpinene	Williams, 1972	γ-cadinene	Kerslake, 1984
γ-terpinene	Williams, 1972	β-cadinene	Kerslake, 1984
terpinolene	Williams, 1972	β-elemene	Derbsey *et al.*, 1980
β-elemene	Williams, 1972	δ-elemene	Le Quéré and Latrasse, 1990
octanone-3	Derbsey *et al.*, 1980	β-guaiene	Le Quéré and Latrasse, 1990
α-copaene	Derbsey *et al.*, 1980	γ-elemene	Le Quéré and Latrasse, 1990
α-humulene	Derbsey *et al.*, 1980	β-cubebene	Le Quéré and Latrasse, 1990
tricyclene	Kerslake, 1984	aromadendrene	Le Quéré and Latrasse, 1990
α-thujene	Kerslake, 1984	α-gurjunene	Le Quéré and Latrasse, 1990
propylbenzene	Kerslake, 1984	γ-muurolene	Le Quéré and Latrasse, 1990
isopropylbenzene	Kerslake, 1984	bicyclogermacrene	Le Quéré and Latrasse, 1990
β-thujene	Kerslake, 1984	ledene	Le Quéré and Latrasse, 1990
1-ethyl-2-methylbenzene	Kerslake, 1984	γ-cadinene	Le Quéré and Latrasse, 1990
1,2,3-trimethylbenzene	Kerslake, 1984	δ-cadinene	Le Quéré and Latrasse, 1990
1-methyl-2-ethylbenzene	Kerslake, 1984	viridiflorene	Le Quéré and Latrasse, 1990
trans-β-ocimene	Derbsey *et al.*, 1980	germacrene B	Le Quéré and Latrasse, 1990

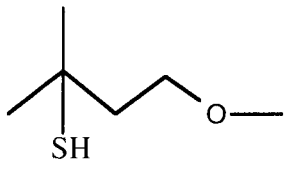
Table 1. Components Identified in Blackcurrant Buds

alcohols	
geraniol	Glichitch and Igolen, 1937; Latrasse, 1969
cis-sabinene hydrate	Le Quéré and Latrasse, 1990
trans-sabinene hydrate	Le Quéré and Latrasse, 1990
trans-p-menth-2-en-1-ol	Le Quéré and Latrasse, 1990
sabinol	Glichitch and Igolen, 1937
α-terpinol	Fridman, 1971
linalool	Latrasse, 1969
citronellol	Williams, 1972
4,6-menthdien-8-ol	Williams, 1972
octane-1-ol-3	Derbsey *et al.*, 1980
terpinene-4-ol	Williams, 1972
iso-butanol	Kerslake, 1984
n-butanol	Kerslake, 1984
pentan-2-ol	Kerslake, 1984
2 methyl butan-1-ol	Kerslake, 1984
3,5,5-trimethyl hexanol	Kerslake, 1984
2-ethyl hexanol	Kerslake, 1984
cis-p-menth-2-ene-1-8-diol	Kerslake, 1984
trans-piperitol	Kerslake, 1984
cis-p-menth-2-en-1-ol	Le Quéré and Latrasse, 1990
p-mentha-1,5-dien-8-ol	Le Quéré and Latrasse, 1990
p-mentha-1,3-dien-8-ol	Le Quéré and Latrasse, 1990
(+)-spathulenol	Le Quéré and Latrasse, 1990
isospathulenol	Le Quéré and Latrasse, 1990
α-cadinol	Le Quéré and Latrasse, 1990
p-menth-2-en-7-ol	Le Quéré and Latrasse, 1990
cis-piperitol	Le Quéré and Latrasse, 1990
isospathulenol isomer	Le Quéré and Latrasse, 1990
sterols	
campesterol	Derbsey *et al.*, 1980
stigmasterol	Derbsey *et al.*, 1980
β-sitosterol	Derbsey *et al.*, 1980
δ-5-avenasterol	Derbsey *et al.*, 1980
δ-7-stigmasterol	Derbsey *et al.*, 1980
δ-7-avenasterol	Derbsey *et al.*, 1980
epoxides	
caryophyllene epoxide	Kerslake, 1984
humulene epoxide	Kerslake, 1984
cis-limonene 1,2-epoxide	Le Quéré and Latrasse, 1990
nitriles	
(E)-3-hydroxy-2-methyl-butyrnitrile	Nishimura et al., 1987
(E)-2-(hydroxymethyl)-2-butenonitrile	Nishimura et al., 1987
thiols	
4-methoxy-2-methyl-2-butanethiol	Riguard *et al.*, 1986

aldehydes	
benzaldehyde	Kerslake, 1984
acids	
acetic acid	Glichitch and Igolen, 1937
hardwickic acid	Derbsey *et al.*, 1980
O-acid	Derbsey *et al.*, 1980
esters	
β-citronellyl acetate	Latrasse, 1969
citronellyl acetate	Williams, 1972
bornyl acetate	Williams, 1972
methyl palmitate	Williams, 1972
citronellyl formate	Kerslake, 1984
4-terpinyl acetate	Kerslake, 1984
β-terpinyl acetate	Kerslake, 1984
geranyl acetate	Kerslake, 1984
methyl undeconoate	Kerslake, 1984
linalyl acetate	Le Quéré and Latrasse, 1990
α-terpinyl acetate	Le Quéré and Latrasse, 1990
neryl acetate	Le Quéré and Latrasse, 1990
ketones	
menthone	Kerslake, 1984
2-undecanone	Kerslake, 1984
umbellulone	Le Quéré and Latrasse, 1990
carvone	Kerslake, 1984
cryptone	Le Quéré and Latrasse, 1990
ethers	
1,8-cineole	Le Quéré and Latrasse, 1990
ethers	
1,8-cineole	Le Quéré and Latrasse, 1990
phenols	
phenol	Glichitch and Igolen, 1937
p-cymene-8-ol	Kerslake, 1984
m-cymen-8-ol	Le Quéré and Latrasse, 1990
β-napthol	Glichitch and Igolen, 1937

Table 1. continued. Components Identified in Blackcurrant Buds

Obviously then blackcurrant oils are a complex mixture, however the contribution of each to the aroma impact varies widely depending on odour thresholds and concentration. The naturally occurring thiol, 4-methoxy-2-methyl-2-butanethiol (1), has been identified as one of the most important compounds contributing to the aroma of blackcurrant extract (Rigaud *et al.*, 1986). Despite being present in only very small amounts, it contributes what is described as a 'catty' note.



1

Not exclusive to blackcurrants, this chemical has been detected in clary sage (*Salvia sclarea* L.) (Van de Waal *et al.*, 2002) green tea and in virgin olive oil (Guth and Grosch, 1991)

Many other components also contribute to the characteristic odour of the oil extracted from blackcurrant buds. Monoterpenes afford green, resin-like notes, monoterpene alcohols generally permeate floral notes while related acetates have been found to be particularly important for their characteristic floral or lemony notes (Latrasse *et al.*, 1982). Bicyclogermacrene, sesquiterpene alcohols and oxides such as spathulenol, caryophyllene oxide and isospathelenol afford conifer-like odours and are found in higher amounts in aromatic varieties such as Noir de Bourgogne. All are considered important compounds contributing to the blackcurrant oil quality (Latrasse and Lantin, 1974; Le Quéré and Latrasse, 1986; Le Quéréand Latrase, 1990). Maximising the recovery of quality volatile components and maintaining their levels through the harvest and extraction processes then must be a primary objective.

VARIETAL DIFFERENCES WITH REFERENCE TO THE NEW CLONES

In Tasmania, Australia, the main commercial blackcurrant cultivar is White Bud, which is a selection from the widely-grown European cv. Baldwin. As stated the naturally occurring thiol, 4-methoxy-2-methyl-2-thiol butane, has been identified as one of the most important compounds contributing to the aroma of blackcurrant extract (Rigaud *et al.*, 1986). Higher levels of 4-methoxy-2-methyl-2-butanethiol have

been detected in some plants of cv. White Bud grown at Bushy Park in Southern Tasmania. The varietal differences in the composition of the essential oil extracted from blackcurrants buds have been used to characterise families (Latrasse and Lantin, 1974). Levels of sabinene, δ–3-carene, β-phellandrene and terpinolene were found to be discriminating features. The terpene profiles of the phenotypes of cross fertilised cultivars were used to consider common biosynthetic pathways and extrapolate to possible genetic relationships across cultivars (Latrasse and Lantin, 1976). Geographical distinctions were assessed and an abundance of terpinene-4-ol was recorded for some varieties which had similar origin (Latrasse *et al.* 1982). The differences in extracts from 10 cultivars grown in Southern Tasmania have been determined (Kerslake and Menary, 1985). Kerslake and Menary found that the relative proportions of sabinene, δ–3-carene and terpinolene were not sufficient to distinguish cv. Baldwin from other selections as proposed by Latrasse and Lantin (1976). The differences in terpene composition reported in the two studies may have been a result of dissimilar extraction protocols and/or climatic differences affecting the phenotype. Indeed the oxygenated compounds content also varied from 5 to 10 % of the oils depending on the cultivars. These polar fractions contained the most odorous volatile compounds and exhibit the characteristic blackcurrant odour (Latrasse and Lantin, 1982). The major compounds were terpinen-4-ol (0.4 to 4.5 %), spathulenol (0.0-2.7 %), β-caryophyllene (0.2-1.5 %) and bornyl acetate (trace to 2.0 %) (Le Quéré andLatrasse, 1990). Considering the moderate polarity of the 4-methoxy-2-methyl-2-butanethiol afforded by the methoxy and the thiol group it may well be possible that the hydroalcoholic infusions eluted on silica with dichloromethane may have contained the endogenous thiol.

Detailed studies on the hydrocabon chemotypes identified 6 monoterpenes, the relative concentrations of which could be used to group 11 cultivars into 4 distinct groups. Using this system the majority of cultivars from France were clearly delineated from those with UK parentage (Kerslake *et al.*, 1989). The relative abundances of 4 sesquiterpenes were used to establish a chemotype formula used to further discriminate between the cultivars. The relative distribution of 24 major compounds including monoterpenes, oxygenated monoterpenes, sesquiterpenes and oxygenated

sesquiterpenes was used to distinguish 23 blackcurrant cultivars of diverse origin and parentage (Latrasse *et al.*, 1990). Each of the cultivars was described with chemotaxonomical formulations with similarities in varietal groups often coinciding with close parentage.

The increased level of 4-methoxy-2-methyl-2-butanethiol in the clonal material propagated at the University of Tasmania may be accompanied by characteristic variations in the levels of other volatiles. Elucidation of the chemical profile may establish the relationships between the high thiol containing selections, White Bud and other favourable varieties.

ACCUMULATION OF OIL AND THE EFFECT OF DORMANCY

The accumulation of oil in blackcurrant buds and maintenance of those levels through dormancy has been studied (Kerslake, 1984; Poulter, 1991). The oil is produced in glandular scales which consists of a basal cell, a stalk several cells in length and a head consisting of a number of secretory cells forming a flattened scale reminiscent of those found in Hop *Humulus lupulus* and in the genera *Thymus, Mentha and Stureja* (Haberlandt, 1928). The bud consists of an overlapping series of leaf scales, transition leaves, bracts and flower and leaf primordia with the density of glands greatest on the upper regions of the leaf scale and decreasing towards the base of the leaf scale and the leaf primordia (Poulter, 1991). Oil gland size increased most rapidly prior to the maturation of fruit at a time of rapid leaf growth corresponding to the period of maximum canopy cover, however oil accumulation was most rapid after photosynthate could be re-directed from leaf growth to secondary metabolism (Kerslake, 1984). The rate of oil accumulation slowed in autumn and spring as the average daily incident of solar energy available for photosynthesis declined. Terpenoid formation occurs only when cells are metabolically active (Gershenzon and Croteau, 1989) so that it would be expected that fluctuations in oil content during the period of dormancy would be minimal. The oil composition in dormant buds was studied at the University of Tasmania in 1990 and 1992 (Poulter, 1991; Poulter, 1992). Poulter found that concrete yields remained relatively constant over the dormant season until just prior to bud burst when there was a doubling in concrete yield over a period of 50 days. The

levels of 4-methoxy-2-methyl-2-butanethiol were found to be high in autumn but decreased during early winter. A subsequent rise was again recorded in late winter before decreasing as bud burst approached. The non-linear response of the detector in use (flame photometric detection) and the lack of 1 : 1 response between the target thiol and the internal standard octanethiol may account for some of the inconsistencies in determining the levels of the thiol in this study. The level of diterpene acids decreased in blackcurrant buds in autumn but remained constant throughout dormancy and constituted 35 % of the weight of concrete extracted. This level decreased to approximately 13 % at the time of bud burst. The levels of volatiles were reported to increase up until bud burst in the same study. These results indicate that significant changes in oil composition do occur throughout dormancy. The coordination and timing of the harvesting of buds in Tasmania from July to late August is limited by the availability of the mechanical harvester (pers. comm. Essential Oils of Tasmania Pty. Ltd.) however, there appears to be some scope to adjust harvest time to maximise desired oil composition and yield. This is particularly relevant in the context of the effect of bud burst on oil quality as elucidated by Kerslake (1984) and by Poulter (1991). Kerslake monitored the yield and composition in the final stages of dormancy through to leaf initiation. It was found that a period of increased oil biosynthetic activity was evident during this period with interconversions resulting in the decrease then increase in monoterpenes such as δ-3-carene and α-terpinolene and in γ-terpinene and α-thujene. The levels of limonene increased as α-terpineol declined. The interconversion of α-terpineol to limonene has been reported (Manitto, 1981). The inability of enzymatic preparations to dehydrate α-terpineol to limonene or terpinolene (Croteau, 1987) led to reservations regarding the early speculation that α-terpineol was the cyclic parent to both reduced species. Limonene and terpineolene have been shown to be simple cyclisation products of geranyl pyrophosphate. None the less, as proposed by Kerlake (1984), limonene may have provided the substrate to further conversions to overcome a lack of photosynthate availability following dormancy. This in turn may have been overcome as the amount of photosynthetic leaf surface increased as bud break progressed. It was also proposed that as α-pinene and β-pinene are formed from the interconversion of geraniol, nerol and linalool (Manitto, 1981)

and the decrease in the levels observed up until 60 % bud burst was also due to the lack of photosynthate for oil synthesis.

Kerslake undertook the study into the effect of bud burst on oil quality in 1984, prior to the identification of the component to which the catty note in the aroma profile is attributed; 4-methoxy-2-methyl-2-butanethiol (Rigaud *et al.*, 1986). However he observed that the strength of the catty note increased as buds broke dormancy until the level overwhelmed the subtlety of the fruity, fresh top notes. This occurred at 65-70 % bud burst such that the benefits of increased yield from expanding buds would be offset by increased extraction costs and poor aroma impact. What is evident is that the period of dormancy and bud burst is not one of compositional stability. Monoterpenes which are stored in, or exposed to, physiological active tissues can undergo metabolic turn over which is highly dependent on the balance between photosynthesis and utilisation of photosynthate (Croteau, 1986). In dormant buds this would expected to be low as tissues would physiologically inactive and photosynthetic processes should be minimal. None the less manipulation of harvest time has the potential to significantly alter the quality and yield of blackcurrant extract.

HARVEST TECHNOLOGY

Mechanised bud harvesting is now standard in Tasmania. Prior to the development of the machine harvester, buds were cut from the cane by hand. This was laborious and slow with an experienced worker taking approximately 5 hours to harvest 1 kg of buds such that it required around 200 hours to produce 1 kg of product (assuming a yield 2.4 %) (Derbesy, *et al.*, 1980). A prototype mechanical harvester was trialed in Tasmania (Kerslake, 1984) with some success though the extract produced from mechanically harvest buds were organoleptically inferior to extracts from hand-cut buds. Subsequent designs achieved a 84 % recovery of buds compared to 94 % for hand-cut with an expected harvesting capacity of up to 2.5 hectares producing 100 to 150 kg of bud or 1 hectare per day of high density plantations (150 to 250 kg) (Menary, 1986). A later study conducted in 1990 at the University of Tasmania investigated the use of a hammer mill to break the structure of blackcurrant buds prior to extraction as opposed to the use of a specialised roller machine which pressed buds

through an 0.8 mm gap (Menary, 1990). The decrease in yield of concrete and volatiles effected by crushing of buds with the hammermill validated the continued use of the roller. However, the superior quality of extracts from hand-cut buds was again confirmed. The 1990 study also investigated the proportion of buds in machine-harvested material. Whole buds were separated by hand from the 'other' material that consisted of loose bracts, bark and broken buds. Additional bud samples were harvested from the canes by hand and extracted. Intact buds constituted 60 % of the machine harvested material and yielded 4.5 % concrete whilst the remaining 40 % 'other' material yielded 2.04 %. The yield of extract from buds cut by hand from the blackcurrant cane was 3.86 %. Although the intent of the study was to assess the efficiency of the machine harvester the amount of extraneous material co-harvested with the buds, it may be assumed, accounts for 40 % of the extraction costs whilst returning a yield of only 2.04 %. Although the non-viability of the traditional hand-cut methods entrenches the continued use of the machine-harvester, improved yield and quality may be obtained by removal of miscellaneous material from harvested buds. In addition the damage to bud structure by machine-harvesting may initiate metabolic, catabolic and oxidative alteration of oil composition.

POST-HARVEST SYNTHESIS – Metabolism and catabolism

The building blocks of many of the aromatic volatiles fundamental to blackcurrants are terpenes which are structural variations based on the five carbon isoprene unit. When two of these 5-carbon units are joined, they form monoterpenoids (C_{10}), when three are joined, they form sesquiterpenoids (C_{15}). The many variations of structural and stereochemical isomers and the introduction of oxygenated moieties all contribute to a myriad of olfactory stimuli with the aroma profile. With the vast array of aroma active chemicals synthesised within plants almost all arise from one of three biosynthetic pathways, the acetate, mevalonate and shikimate. The terpenes are wholly derived from the mevalonate pathway, common to the synthesis of many biological molecules constructed of isopentenoid units including chlorophylls, gibberellins, carotenoids, steroids and natural rubber (Croteau, 1984). This pathway is integral to the synthesis of building blocks fundamental to plant function as well as to the formation of secondary metabolites.

Terpenoid accumulation in plants is generally restricted to specialized secretory structures close to the plant surface to allow for release of volatiles. Indeed the oils glands are the major site of terpenoid biosynthesis (Croteau, 1984). Compartmentalisation serves to limit the risk of toxicity to the plant (Gershenzon, 1994). In addition many terpenes are accumulated as non-volatile consitituents such as terpene diphosphates and flavorless glycosides which facilitates transportation by increased water solubility and decreased reactivity (Croteau, 1987; Winterhalter and Skouroumounis, 1997). Since the first detection of glucoconjugated forms of monoterpene alcohols in rose petals 25 years ago (Francis and Allcock, 1969) these precursors have been identified on 58 plant families (reviewed by Winterhalter and Skouroumounis, 1997). In many cases glycosidically bound flavors was found to exceed the amount of the free aroma in a ratio range of 2:1 to 5:1. The many aromatic volatile compounds bound to glycosides can be released during storage, pre-treatment or by enzyme and acid catalysed reactions (Crouzet and Chassagne, 1999).

The activation of enzymatic and oxidative processes has the potential to improve or detract from the quality of extracts from plant material by releasing aglycones from glycoconjugates or oxidising and metabolising existing terpenoids. Post-harvest treatment and storage conditions of buds can affect oil quality as a result of the activation of plant response mechanisms to damage, de-compartmentalization of substrates and enzymes and their exposure to oxidative conditions.

The auto-oxidation and polymerisation of the monoterpene fraction of the essential oil of blackcurrant buds have been investigated (Latrasse and Demaizieres, 1971). The five terpenes most readily oxidised included α-phellandrene, δ-3-carene, β-phellandrene, β-mycrene and an unidentified component. The monoterpenes are considered important for their floral and lemony notes. The degradation of lipids to volatile constituents in black leaf tea after rolling and fermentation has been studied (Selvendran *et al.*, 1978). Selvendran found that mechanical damage increased the headspace concentration of C_6 aldehydes and postulated that the breakdown of membrane lipids may initiate the formation of volatile compounds which contribute to the flavour of black tea. The storage of black tea leaves under oxidative conditions

was shown to increase the concentration of 6 volatile components (Springett and Williams 1994). The production of volatile aliphatic and phenolic components was accelerated by moisture and heat in stored black tea (Stagg, 1974). The flavours of yellow passion fruit (*Passiflora edulis* f. *flavicarpa*) were also increased during storage (Narain and Bora, 1992). The changes in volatile content effected by harvesting of tea rose (*Rosa damascena*), narcissus (*Narcissus tazetta*), osmanthus (*Osmanthus fragrans*) and spearmint (*Mentha spicata*) have been demonstrated (Mookherjee *et al.*, 1986; Mookherjee *et al.*, 1989). Mookherjee showed that purging with nitrogen gas altered the composition of volatiles emitted. The changes in distilled essential oil content of rose effected by post-harvest treatments have been examined. The storage of chilled plants in anaerobic conditions protects plants from deleterious effects but anaerobic conditions have also been attributed to an increase in essential oil of rose possibly by inhibition of oxidative reactions (Tyutyunnik and Ponomaryova, 1977). The effects of post harvest treatment on the volatile components of boronia flowers (*Boronia megastigma*) has been studied extensively (Mactavish and Menary, 1998a; MacTavish and Menary, 1999; MacTavish and Menary, 1999; Mactavish and Menary, 2000). Floral volatiles were increased by up to 300 % in aerobic conditions in open fresh undamaged flowers. All these studies emphasise the considerable potential for the alteration and increase in yield of many important volatile components by manipulation of post-harvest conditions. Indeed in a study conducted at the University of Tasmania on blackcurrant buds, which were normally frozen intact prior to extraction, were crushed by a mechanised roller prior to freezing (Menary, 1990). Although this was undertaken with view to stream lining the bud extraction process oil yields from blackcurrant buds were significantly affected by their condition prior to storage and by the length of time for which they were stored. The crushed material was stored at -20°C and small samples were withdrawn at intervals and extracted to determine the % yield of concrete. It was observed that the bulk source material yielded 2.3 % whilst the stored crushed material that were extracted in smaller quantities, had declined from 1.9 % at 9 days of storage down to 1.4 % after 42 days. The chemical composition of the extracts was not profiled.

It must be proposed that when blackcurrant buds are stripped from the stem and crushed prior to extraction, the enzymes of terpenoid biosynthesis may no longer be compartmentalised in cell structures and the terpenoids themselves are exposed to effects of oxidation, chemical alterations and volatilisation in the harvest environment. Freezing of the buds is undertaken to retard enzymatic and non-enzymatic processes, thereby preserving the quality of products. However, freezing also has the potential to activate enzyme systems. As water freezes the solutes in the remaining liquid becomes more concentrated, the pH changes and cell injury by ice crystals can cause leakage of cell contents, facilitating interaction of enzymes and substrates (Finkle, 1971). The freezing and structural damage associated with commercial harvests, then, can result in significant changes in extract composition. Therefore the manipulation of harvest and storage conditions must present many opportunities to alter the extract composition of blackcurrant buds.

EXTRACTION TECHNOLOGY

The first recorded extract oil of blackcurrant was introduced to the market around 1925 by the firm Dhumez, Grasse (Dumont, 1941). Cold benzene yielded 2.4 to 3.0 % by weight of bud of a semi-crystalline dark green concrete which was further characterised by producing steam distillate and instigating fractionation and chemical derivatisation techniques to tentatively identify some of the extract components (Glichitch and Igolen, 1937). Benzene was again used as the solvent for preliminary extractions in subsequent research that again focused on characterising and identifying the composition of blackcurrant buds (Chiris, 1937; Latrasse, 1969). Indeed the extract produced within the French blackcurrant industry, that has always been regarded as being of premium quality, used benzene as the extracting solvent (Thomas, 1979), however more recently this was changed to hexane as a result of concerns regarding the carcinogenic properties of benzene. Following a study tour to France in 1986 Kerslake detailed a traditional recipe for the production of blackcurrant concrete using 5 washes of benzene at a bud weight to solvent ratio of 1:2.7 at a temperature of 50°C for 2 hours (Kerslake, 1986).

Although the solvents used to elucidate the chemistry of blackcurrant buds have included carbon tetrachloride (Latrasse and Lantin 1977), pentane (Latrasse and Lantin 1974) and petroleum ether (Kerslake, 1984; Kerslake and Menary, 1985) there has been very few publications that compare the relative composition and yield of different extracting solvents. These types of studies are most likely to have been conducted 'in house' during the commercial development of the blackcurrant bud industry within each country. At the University of Tasmania, however, a series of extraction experiments using hexane, petroleum, ethyl ether, methanol and liquid carbon dioxide was undertaken to determine if a product of higher quality could be produced (Kerslake, 1984). The use of methanol increased the yield of sesquiterpenes compared to those extracted with petroluem ether or hexane however the product had an overall flat impression and lacked the cattiness associated with the French product. Of the solvents tested, petroleum ether was found to produce oils most like the French products. Liquid carbon dioxide extractions were undertaken in two ways. In a laboratory scale experiment liquid carbon dioxide was produced using a pressurised container fitted with a cold finger condensor and introduced into a standard glass Soxhlet extractor. A second liquid carbon dioxide extract was produced in a semi-commercial pilot plant. Both were compared to a petroleum ether extract and a vacuum distillate of petroleum ether extracted concrete. The yield of two extracts produced using carbon dioxide were similar, however the extract produced in the semi-commercial plant was lemon yellow compared to the green waxy aromatic material produced using the Soxhlet extractor. Both extracts were considered to be superior to all other products, retaining a freshness and strength of blackcurrant aroma. However, the instigation of fundamental changes to the infrastructure of the Tasmanian industry was beyond the scope of this study and continued research into this potential extract technnology was not to be pursued.

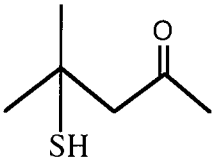
Despite the findings of Kerslake that the extract produced using methanol based extraction had an overall flat impression, previous researches had found that the extracts produced when buds were homogenised under approximately three times their weight of methanol and extracted with pentane were organoleptically superior to those obtained using pentane alone (Williams, 1972). The steeping of material in alcoholic

solutions has been shown to reduce enzymatic activity (Tressel *et al.*, 1970). Yet the use of alcohols as extracting solvents has not been regarded as appropriate in many situations as it solvates water and water-soluble compounds such as pigments and sugars (Guenther, 1972). However the partitioning of the extracted components back into non-polar solvents as undertaken by Williams re-introduces the selectivity of the solvent. Pentane is also more readily removed by rotary vacuum evaporation to produce the final product. Interestingly Kerslake also recounted that through personal communication he was aware that Pernod-Ricard produced an ethanol fruit extract that was incorporated directly into the French Liquer 'Cassis de Dijon'. The potential benefits that may be associated with the inclusion of alcohol in extraction methods for the production of blackcurrant extracts, warrants some investigation. As detailed in the section on post-harvest synthesis the oxidation and continued enzymatic activity of biological material can result in the depletion of quality components. The introduction of an alcohol may serve to, not only retard the loss of components, but also to aid the saturation of solvent into bud structure as non-polar solvents such as petroleum ether do not penetrate plant material with a high moisture content (Gorgiev and Tsvetkova, 1977). The penetration of plant material may also be facilitated by the breaking of bud structure by mechanical means. The application of a specialised roller machine which pressed buds through an 0.8 mm gap has been shown to increase blackcurrant oil yields (Menary, 1990). Extract yield from *Boronia megastigma* (Nees) was increased by 90 % by rolling frozen flowers prior to extraction (MacTavish and Menary, 1998b). The chopping of coriander herb prior to extraction also improved essential oil yield and resulted in lower levels of aldehyde and increased levels of alcohols (Smallfield *et al.*, 1994).

SYNTHESIS AND BIOSYNTHESIS OF THIOLS IN BLACKCURRANTS

Although carbon disulphide, dimethyl sulphide and dimethyl disulphide had been detected in blackcurrant (Von Sydow and Karlsson, 1971) 4-methoxy-2-methyl-2-butanethiol (1) was the first sulphur containing chemical to be directly correlated to the 'catty' note characteristic to blackcurrant extracts (Rigaud *et al.*, 1986). This chemical has been identified as a distinguishing feature of quality extracts and maximising of the thiol concentration is a primary focus to improve the quality of

blackcurrant extracts. Research into the characteristic 'catty' odour reminiscent of tomcat 'aged' urine was continued at the Research Laboratories at Grasse Cedex in France (Joulain and Laurent, 1989). Joulain reported that research analytical chemists had proposed to both perfumers and flavourists several good synthetic substitutes including 4-mercapto-4-methyl-2-pentanone (2) and 8-mercaptomentan-3-one (3).



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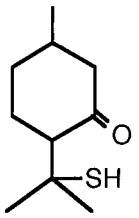

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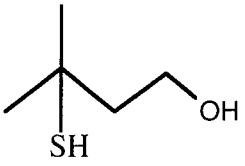

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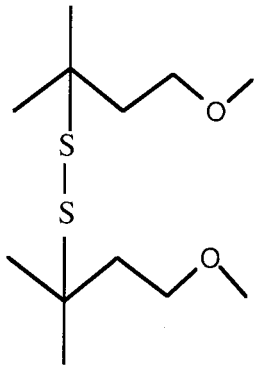
However it was reported that of the 33 sulphur chemicals Joulain investigated only 4-methoxy-2-methyl-2-butanethiol (1) and 2-methyl-thiol-butanol (4) displayed the characteristic blackcurrant or 'catty odour at proper concentration (10 - 1000 ppb range). The similarity of the chemical structures of (1) and (4) supported the findings that the tertiary mercapto amyl substructure to be the dominant feature for determining the 'catty' note (Polak, 1973). Joulain detected both 4-methoxy-2-methyl-2-butanethiol in leaves, berries and buds of blackcurrant and was successful in detecting the disulphide, (5) in buds. The extraction used a soxhlet apparatus with dichloromethane and was followed by silica gel preparative layer chromatography. The presence of the thiol and the disulphide was established using gas chromatography in the selected ion monitoring mode. Joulain referred to felinine (6), isolated from cat urine (Westall, 1953), as a condensation product of prenol (7) and cysteine which may lead one to consider the isopentyl moiety in felinine as part of a possible terpene-like biogenetic pathway.



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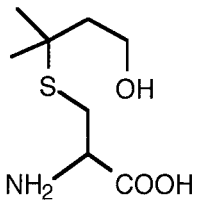

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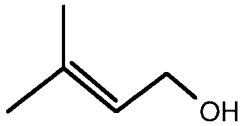

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The release of the offensive odour of cats urine develops on ageing. Joulain suggested this phenomenon very likely originates from the degradation of odourless felinine by common microorganisms in combination with oxidation by ambient air. Following this line of thought Joulain proposed that the formation of sulphur chemicals in blackcurrant extracts may proceed through a similar pathway involving a precursor possessing an S-aminoacid moiety.

Indeed the presence of 4-methoxy-2-methyl-2-butanethiol in other products such as Spanish virgin olive oils (Guth and Grosch, 1991; Guth and Grosch, 1993; Reiners and Grosch, 1999) and in clary sage flowers (Van de Waal *et al.*, 2002) has been reported. Van de Waal, 2002 suggested that the route to 1-methoxyhexane-2-thiol may involve a *Michael* addition of a S-nucleophile to hex-2-enal followed by reduction of the aldehyde and methylation of the intermediate, although no 2-mercaptohexanal was perceived in sage fractions.

Odours reminiscent of blackcurrants were detected by in extracts of Sauvignon wines prompted research into thiols within the viticulture industry (Darriet *et al.*, 1991). Darriet led the research into possible precursors present in non-fermented grape juice that cleave to produce 4-mercapto-4-methylpentan-2-one during fermentation of Sauvignon must (un-fermented grape juice) (Darriet *et al.* 1993). The studies of thiol functions present into Sauvignon blanc wines resulted in the identification of 3-mercapto-3-methylbutan-1-ol (4), amongst other flavour-active mercapto-alcohols (Tominaga *et al*, 1998a). The amplification of the Sauvignon blanc grape aroma during alcoholic fermentation was attributed to the release of flavour-active volatile thiols from the corresponding S-cysteine conjugates with the hypothesis verified by

the mass spectrometry of trimethylated derivatives of the otherwise non-volatile amino acid conjugates (Tominaga *et al.*1998b). In addition crude extracts containing sulphur flavour precursors were extracted from 45 L of must by adsorption chromatography on C_{18} silica and eluted with 1 % ethanol. The extracts were then subject to the action of a cell-free extract from *Eubacterium limosum* which has a cysteine conjugate β-lyase (Larsen and Stevens, 1986) and after a 15 minute incubation at 30°C volatile thiols were released. No volatiles were released if the bacterial extract was de-activated by heating and in view of the substrate specificity of the β-lyase it was envisaged that the thiols were produced from S-cysteine precursors.

The research team at the Faculté d'Œnologie de Bordeaux at Université Victor Segalen, France, went further to develop assays to measure the aromatic potential of grapes and wine musts by assaying the cysteinylated precursors of volatile thiols (Peyrot des Gachons *et al.*, 2000; Murat *et al.*, 2001).

Elucidating the chemistry of thiol production in blackcurrants would underpin research into the conditions required to maximise endogenous biosynthesis. In addition the development of assays similar to those developed at the Faculté d'Œnologie de Bordeaux at Université Victor Segalen could be used for identifying the time of precursor synthesis and sequestering as well as provide for an indicator to the optimium time of harvest to maximise thiol concentration.

2. MATERIALS & METHODS

Section 2.1. HARVEST AND EXTRACTION TECHNOLOGY

Background to Materials and Methods.

The dominance of the French in international trade of blackcurrant extracts is largely attributable to the pungency of the extract they produce and that in part has been conferred by high levels of the endogenous thiol 4-methoxy-2-methyl-2-butanethiol. In France hand harvesting has been replaced by mechanical methods. As with most commercially sensitive information, the extracting solvents used, and the finer details of the extraction processes are confidential. Despite the large number of variables in the harvest and extraction processes that may be manipulated, the impetus remains to secure a viable market share by improving the quality of the Australian product. In section 2.1, a series of experimental protocols that are to be used in preliminary investigations are established. Once protocols are established the fundamental questions addressed with regard to the extraction of blackcurrant buds are:-

- Does the amount of debris co-harvested with the machine harvested buds compromise the efficiency of extraction and the quality of the final product?
- What effect does the method of maceration have on the yield and type of components extracted?
- What solvents are the most efficient in extracting quality components from blackcurrant buds?
- Could the loss of components be retarded by reducing the potential for oxidation during the extraction process?

Having identified protocols that were likely to improve the quality and yield of extracts the adaptation of laboratory based processes to industrial scale operations was undertaken. Finally in this section, having maximized the levels of quality components in the final extract, it is pertinent to investigate whether some of the components are lost during storage of the extract and determine the effectiveness of antioxidants in reducing such losses. Flow charts outlining the experimental approach are detailed in appendix B.

In section 2.2 the study moved to investigate the effect on extract yield and quality of the time of harvest and the storage of buds. The compositional variation between the standard commercial variety, White Bud and a new high thiol clonal variety was also established. Section 2.3 deals with some of the underlying chemistry in the synthesis and biosynthesis of 4-methoxy-2-methyl-2-butanethiol.

2.1. General analytical methods

Solvents

Unless otherwise specified the solvents used in the extractions were as follows

- petroleum ether (boiling point, 40 - 60°C) and n-hexane was supplied from BP Australia and redistilled prior to use. Solvents were tested for impurities and residues by gas chromatograph. Hexane used for analysis was from Mallinckrodt. Ethanol and ethyl acetate were from Mallinckrodt and both were ChromAR, HPLC grade.

Extraction Methods

Standard extraction and analyses methods are common to many of the experiments undertaken. In pursuit of efficient presentation, the basic parameters used in analyses by gas chromatography (GC) are presented in section 2.1.1.ii and 2.1.1.iii. A large proportion of blackcurrant extract is composed of carboxylic acids that are not amenable to gas chromatography and require derivatisation. All extracts to be analysed by GC coupled to a flame ionization detector (FID) were methylated with diazomethane prior to injection.

The large number of extractions envisaged necessitated the adaptation of the extraction protocols. It was not practical to produce blackcurrant extract for every experiment. Two basic methodologies were employed, with variations described within each experiment. The first produced 'solvated extracts'– extract components remained in the solvent with which they were extracted such that the final product, blackcurrant extract, was not produced. The solvent containing the extracted components was sub-sampled and analysed directly. This provided for rapid sample throughput in experiments that generated large numbers of samples. The second method was for the

analyses of blackcurrant extracts produced after the extracting solvent was removed by rotary vacuum evaporation (RVE). This method provided for the complete assessment of extracts including aromatic impact and extract yield and consistency. Experiments to relate the two methods are established in section 2.1.2.

2.1.i. Preparation of diazomethane derivatising solution

Diazomethane (CH_2N_2) solution was prepared from N-methyl-N-nitrosourea (supplied by Sigma, St Louis, USA) in aqueous KOH. In a 250 mL Erlenmyer flask 40 mLs of a 40 % KOH (w/v) and 50 mL of diethyl ether (Fluka Chemika, HPLC grade) was placed on a magnetic stirrer and cooled to 5°C in an ice/water bath. N-methyl-N-nitrosourea (3.0 g) was added over a period of 5 minutes. The suspension was stirred for 20 to 30 minutes the mixture was transferred to a separating funnel. The ether layer was combined with the a 25 mL diethyl ether wash of the aqueous layer and the combined ether layers were stored over beads of KOH for at least 30 minutes at -15°C. The yellow solution was transferred to a screw top Schott bottle and stored at -15°. The reagent was viable for a period of approximately 1 month. To 2 mL GC vials containing 0.5 mL of blackcurrant sub-samples 1 mL of the diazomethane solution was added and left to stand for 10 minutes. After signs of reaction ceased (bubbling) 1 drop of glacial acetic acid was added so that the solution turned from yellow to clear.

2.1.ii. GC FID Analyses

A BPX5 fused silica capillary column (25 m x 0.32 mm i.d., 0.25 μm film thickness) was installed into a Hewlett Packard 5890 Series II GC which was equipped with FID. The carrier gas was instrument grade nitrogen with a head pressure of 10 psi producing a column flow of 0.8 $mLmin^{-1}$. FID analyses were conducted using a 1 μL injection with a split ratio 1:40. Injector and detector temperatures were 260 and 290°C respectively and the temperature ramp was 40°C for 1 minute followed by a 9°$Cmin^{-1}$ ramp to 290°C held for 16.22 minutes. Air, makeup gas and hydrogen flows were set at 70, 25 and 65 $mLmin^{-1}$ respectively.

2.1.iii. GC FPD Analyses

A BPX5 fused silica capillary column (25 m x 0.32 mm i.d., 0.25 μm film thickness) was installed into a Hewlett Packard 5890 Series II GC which was equipped with a

flame photometric detector (FPD). The carrier gas was instrument grade nitrogen with a head pressure of 10 psi producing a column flow of 0.8 $mLmin^{-1}$. FPD analyses were conducted using 3 µL splitless injections with the injector and detector held at 240 and 250°C respectively. An initial temperature of 40°C was held for 2 minutes followed by a 25°$Cmin^{-1}$ ramp to 250°C held for 12.60 minutes. The air flow to the detector was 78 $mLmin^{-1}$, auxiliary gas at 25 $mLmin^{-1}$ and instrument grade hydrogen at 65 $mLmin^{-1}$.

2.1.iv. Solvated Extract Methodology and Analysis

Buds (10 to 50 g) were weighed into conical flasks and 4 x w/v of 5 % hexane in petroleum ether was added. Each flask was spiked with the equivalents of 1.7 μgmL^{-1} octanethiol and 0.8 $mgmL^{-1}$ octadecane, internal standards for flame photometric detection and flame ionisation detection respectively. Samples were pulverized using an ultraturrex (an homogeniser with a rotating shaft blade enclosed within a stainless steel shaft) and allowed to settle for 20 minutes. Aliquots (0.5 mL) of each sample were transferred into 2 mL GC vials. The terpene acids were methylated with diazomethane. Excess reagent was quenched with glacial acetic acid and the sample analysed by GC FID for volatile components and methylated acids. A further 1 mL was sub-sampled into a second GC vial and analysed immediately by GC FPD to quantify the level of 4-methoxy-2-methyl-2-butanethiol in the buds under the conditions described.

2.1.v. Sample Preparation for Analysis of Blackcurrant Extracts

Crystalline blackcurrant extracts were warmed gently in a 30°C oven to provide for a fluid, consistent sample. Aliquots (25 – 30 mg) were transferred to GC vials. Octadecane (Sigma Aldrich) (1 mg) was added to samples to be analysed by GC FID. The terpene acids were derivatised with 0.5 mL of diazomethane solution and left to stand for 10 minutes or until all signs of reaction had ceased. Excess reagent was quenched with glacial acetic acid and the samples analysed by GC FID as described. For GC FPD analyses 25 – 35 mg aliquots of blackcurrant extract were dissolved in 1 mL of 5 % hexane in petroleum ether, spiked with 1.7 µg of octanethiol (Fluka Chemica, Switzerland) and analysed as described in section 2.1.iii.

2.1.1. Sieving Experiment.

In commercial operations blackcurrant buds were mechanically harvested. As a result a proportion of the harvested bulk consisted of cane shards, bark and dried leaves. In this experiment the buds were sieved prior to extraction to assess whether the exclusion of co-harvested debris could reduce extraction costs and improve oil quality.

Freshly harvested buds (14 kg) were introduced into the winnower. Sieve #10 and #7 had mesh with holes of diameters of 9.5 mm and 5 mm respectively whilst the fines were collected through mesh containing 9 holes / square inch. All samples were sub-sampled for dry weight estimates. In all, six samples were frozen at -18°C; the un-sieved buds, sieved buds, #10 and #7 sieve collections, the fines and the winnow fraction. The winnow fraction was a light fibrous material blown into a higher collection terminal as the sieved samples dropped into the main collection bag.

After being frozen for 2 weeks the samples were allowed to thaw for 15 minutes and 50 g sub-samples were taken from each. Samples were extracted in 200 mL of 5 % hexane in petroleum ether and ultraturrexed for 3 x 2 minute bursts to prevent overheating of the machine. The small sticks and pieces of broken canes etc. collected from #7 sieves precluded effective application of the ultra-turrex, whilst the sample from sieve #10 could not be ultra-turrexed at all. The samples were placed on a shaker for 30 minutes then dried down at 40°C, with a final 5 minutes at 60°C. Sub-samples were taken for analyses by GC FID to assess volatiles and by GC FPD for thiol quantification.

2.1.2. Effect on Yield of Methods of Maceration and Solvent Composition.

As discussed, undertaking complete extractions of large sample numbers was not feasible and solvated extracts were sub-sampled without the inclusion of a dry down step. To design an effective extraction method and to be able to relate the results from such sub-sampling techniques to the results obtained from complete extractions, a series of simple extraction experiments were undertaken. In all 5 methods were trialed. The first details the standard extraction with dry down effected by RVE. The remaining four methods produced solvated extracts but investigated the difference in

component yield using varying masceration and solvent protocols. The 5 methods are summarised;

1. Full extraction of rolled buds with agitation using a shaker bath.
2. Buds ground under liquid nitrogen in a mortar and pestle and extracted in hexane.
3. Buds ground under liquid nitrogen in a mortar and pestle and extracted in 5 % hexane in petroleum ether.
4. Buds ground under liquid nitrogen in a mortar and pestle and extracted in 1 % hexane in petroleum ether.
5. Buds pulverised using a stomacher (an extraction machine with a chamber which has metal paddles within, which pummel buds in solvent within re-inforced plastic bags) and extracted in 1 % hexane in petroleum ether.

Method 1. Full standard extraction with production of concrete

Approximately 300 g of the frozen blackcurrant buds were rolled, reproducing the conditions under which concrete is produced commercially. Five 50 g samples were weighed into 500 mL conical flasks and extracted in 3 x w/v 5 % hexane in petroleum ether. The flasks were placed on a shaker bath for 3 hours. This process was repeated a further 3 times with a final extraction of the buds with 2 x w/v solvent with agitation for 2 hours. The extracts were filtered through cotton wool into a pre-weighed 1 L round bottom flask and dried down at 40°C in a rotary vacuum evaporator (RVE). Sub-samples of 20 – 30 mg were taken for analysis using GC FID (with derivatisation) and GC FPD.

Method 2. Extraction in hexane with buds ground with a mortar and pestle

Sub-samples of 5 x 12 g of frozen buds were placed into a stainless steel mortar and ground under liquid nitrogen. The crushed buds were transferred to 250 mL conical flasks and 4 x w/v of 100 % hexane was added. The samples were sonicated for 10 minutes. Octadecane (191 mg) was added to each flask as an internal standard. The extracts were sub-sampled (0.5 mL) into 1 mL GC vials, derivatised using diazomethane and analysed by GC FID.

Method 3. Extraction in 5 % hexane in petroleum ether.

Sub-samples of 5 x 12 g of blackcurrant buds were pulverised under liquid nitrogen and extracted as described for method 2. However the extracting solvent used was 5 % hexane in petroleum ether.

Method 4. Extraction in 1 % hexane in petroleum ether.

Sub-samples of 5 x 12 g of blackcurrant buds were pulverised under liquid nitrogen and extracted as described for method 2. However the extracting solvent used was 1 % hexane in petroleum ether.

Method 5. Extraction in 1 % hexane in petroleum ether using a stomacher.

Sub-samples of 5 x 12 g of blackcurrant buds were pulverised in the stomacher in specialised bags for 15 minutes. The extracting solvent was 1 % hexane in petroleum ether. The samples were sonicated for 10 minutes then allowed to settle for 15 minutes. Octadecane (191 mg) was added and 0.5 mLs of each extraction was transferred to GC vials and derivatised with diazomethane for analyses by GC FID.

2.1.3. Effects of Homongenisation Using an Ultra-turrex.

Previous experiments demonstrated that the use of a mortar and pestle to grind samples was time consuming and returned poor yields. An alternative method was to macerate buds using an ultra-turrex. To inter-relate the two methods the levels of thiols, volatiles, acids and yields of concrete for buds ground under liquid nitrogen before solvent addition was compared to the yields from buds ground in the standard blackcurrant solvent (5 % hexane in petroleum ether) using the ultra-turrex. Each extraction was conducted in triplicate.

Method 1. Grinding in air with a mortar and pestle.

Approximately 30 g of buds were ground whilst still frozen in a stainless steel mortar and pestle under liquid nitrogen. Three replicates of approximately 5 g were weighed into 3 Erlenmeyer flasks and 4 x w/v of 5 % hexane in petroleum ether was added to each. The samples were sonicated for 10 minutes and spiked with 20 mg of octadecane and 44 μg of octanethiol. Samples were left to settle for 10 minutes then 0.5 mL was sub-sampled and derivatised using diazomethane for analysis by GC FID.

Further sub-samples of 1 mL were analysed by GC FPD to quantify the amount of endogenous thiols extracted.

Method 2. Grinding in solvent using an Ultra-turrex.

Approximately 3 x 5 g of blackcurrant buds were placed in a ultra-turrex with 4 x w/v of 5 % hexane in petroleum ether. The samples were homogenised for 15 to 20 second bursts. Samples were fortified with 20 mg of octadecane and 44 µg of octanethiol. After a settling period of 10 minutes, 0.5 mL was sub-sampled from each replicate and derivatised using diazomethane for analyses by GC FID whilst a further 1 mL was sub-sampled to quantify endogenous thiols using GC FPD.

2.1.4. Preliminary Experiments to Include the Steeping of Buds in Ethanol Prior to Extraction.

Improving extract quality by advancing extraction technology was a prime objective in this study. Steeping in ethanol solvates the water component of blackcurrant buds, ensuring effective sample penetration whilst deactivating enzymatic processes. This has the potential to reduce degradation of quality components during the extraction procedure. In the following experiments ethanol is used in the first stages of extraction. The co-extraction of excessive amounts of un-wanted polar components was avoided by the extraction of the ethanol-steeped buds with non-polar solvents via the formation of a partition, thereafter following the procedures already standard in the blackcurrant industry. Preliminary experiments indicated that the formation of a partition between the ethanol/bud mixture and the 5 % hexane in petroleum ether was enhanced by the addition of a small amount of water to ensure a distinctive phase separation between the solvents (data not reported). A third experiment detailed in this section, investigated the application of ethyl acetate in the extraction of blackcurrant buds, as this solvent had been recommended through communications with European counterparts (pers. comm., R.C. Menary). Ethyl acetate was included in the final extraction step to increase the recovery of polar components into the non-polar partition. The three extraction methods undertaken were;

1. using 5 % hexane in petroleum ether as the extracting solvent.
2. steeping the buds in ethanol and partitioning the non-polar components into 5

% hexane in petroleum ether.

3. steeping the buds in ethanol, adding ethyl acetate and partitioning non-polar components into 5 % hexane in petroleum ether.

Method 1. Hexane (5 %) in petroleum ether.

Frozen buds were passed through a rolling machine and 100 g sub-samples were weighed into 500 mL flasks. Solvent (300 mL of 5 % hexane in petroleum ether) was added and the flasks were placed in a shaker bath for 3 hours. The solvent was decanted into round bottom flasks and the blackcurrant buds were re-extracted in 2 x 200 mL of 5 % hexane in petroleum ether with agitation for 2 hours. All the solvent extracts were combined and dried down on the RVE with a final dry down at 40°C for 5 minutes. Sub-samples of the concretes were analysed by GC FID (with derivatisation using diazomethane) and GC FPD.

Method 2. Ethanol based extraction.

Frozen rolled buds (100 g) were homogenised in a blender with 2 x w/v of ethanol (200 mL) for 20 seconds. Solvent (400 mL of 5 % hexane in petroleum ether) was added and the solvents decanted from the buds and filtered through a Buchner funnel with Whatmann #1 filter paper. The filtrate was transferred to a separating funnel and 40 mL of distilled water was added, the mixture was well shaken and the two phases were allowed to separate. The lower aqueous layer was removed and re-extracted in 400 mL of 5 % hexane in petroleum ether. After mixing and separation this process was repeated a third time. The non-polar fractions were combined and dried down on an RVE. Sub-samples of the concretes were analysed by GC FID (with derivatisation using diazomethane) and GC FID.

Method 3. Ethanol/ethyl acetate/water extraction

Frozen rolled buds (100 g) were homogenised in a blender with 2 x w/v of ethanol (200 mL) for 20 seconds. Solvent (300 mL of 5 % hexane in petroleum ether) was added and 200 mL of ethyl acetate. The mixture was filtered through a Buchner with Whatmann #1 filter paper and the filtrate was transferred to a separating funnel. Water (10 mL) was added to effect a partition and the non-polar layer was transferred to a

round bottom flask. The aqueous layer was washed with a further 2 x 200 mL of 5 % hexane in petroleum ether and the washings combined with the non-polar extract in the round bottom flask. The solvent was removed by RVE with a final dry down time of 5 minutes at 40°C. Sub-samples of the concrete were analysed by GC FID (with derivatisation using diazomethane) and by GC FPD.

2.1.5. Further Development of the Extraction Protocol to Include Steeping in Ethanol

To prevent the degradation of important volatile components during the extraction process, rolled blackcurrant buds were steeped in alcohol, prior to extaction using the solvent most commonly used in the blackcurrant industry, 5 % hexane in petroleum ether. This non-polar solvent was used to establish a partition with the polar ethanol/bud extract mixture. Following the removal of the buds the formation of a partition between the ethanol/bud extract mixture and the 5 % hexane in petroleum ether was enhanced by the addition of a small amount of water to ensure a distinctive phase separation between the solvents. In the previous experiment (section 2.1.4. method 2) the extracting, non-polar solvent was only in direct contact with the buds prior to partitioning, with subsequent washes used only to extract components from the polar ethanol layer. Many of the non-polar components may not have moved into the polar extracting solvent and may have still been present in the buds. In this experiment the extract produced when the ethanol mixture is removed from the buds prior to partitioning is compared to extract produced when partitioning was undertaken whilst the ethanol mixture remained in contact with the buds. Also, to ensure the recovery of any components which may not have been extracted in the ethanol, the buds were re-extracted with 5 % hexane in petroleum ether.

In the previous experiment, the 2 x v/w of ethanol to bud ratio was only sufficient to cover the buds. In this experiment the solvent volume is increased to 3 x v/w ethanol to bud ratio. The previous experiment (section 2.1.4) also indicated that the formation of a partition between the ethanol/bud mixture and the 5 % hexane in petroleum ether may not have required the addition of water to ensure a distinctive phase separation between the solvents. In the following experiment water was not added.

Method 1. Partitioning into non-polar solvent whilst still in contact with buds.
Ethanol (300 mL) was added to 100 g of frozen rolled blackcurrant buds and the mixture was homogenised for approximately 30 seconds. Whilst the ethanol was still in contact with the buds, 400 mL of 5 % hexane in petroleum ether was added. After the formation of the partition the non-polar layer was removed and the ethanol / buds were re-extracted a further 2 times with 400 mL of 5 % hexane in petroleum ether. The non-polar extracts were combined and dried down on the RVE at 40°C with a final dry down at 60°C for 5 minutes. The buds were back extracted with 400 mL of 5 % hexane in petroleum ether, sonicated for 10 minutes and filtered. The resulting marc (extract produced from the re-extraction of waste material) was then dried down, weighed and analysed separately.

Method 2. Partitioning into non-polar solvent following removal of buds
Ethanol (300 mL) was added to 100 g of frozen blackcurrant buds and homogenised for approximately 30 seconds. The ethanol extract was filtered using a Buchner funnel with Whatmans No.1 filter paper. The filtrate was transferred to a separating funnel and 400 mL of 5 % hexane in petroleum ether was added. The mixture was well shaken and, after the formation of partition, the non-polar layer was removed and the ethanol layer was re-extracted a further 2 times with 400 mL of 5 % hexane in petroleum ether. The non-polar extracts were combined and dried down on the RVE at 40°C with a final dry down at 60°C for 5 minutes. The buds were back extracted with 400 mL of 5 % hexane in petroleum ether, sonicated for 10 minutes and filtered. The resulting marc was then dried down, weighed and analysed separately.

2.1.6. The Effect of the Introduction of Propylene Glycol into the Extracting Solvent
The low volatility of propylene glycol presented the potential to reduce the loss of low volatile compounds such as the endogenous thiol in blackcurrant buds. In this context the propylene glycol may act as a 'keeper'. Propylene glycol was included in the extracting solvent. Ratios trialed were 1:1 and 0.2:1 propylene glycol to bud weight (v:w) in the ethanol extracting solvent. Duplicate extractions were undertaken for both methods.

Method 1. Extraction with 1:1 propylene glycol to bud ratio.

Blackcurrant buds (100 g) were rolled frozen and placed in a blender. Propylene glycol (Ajax Chemicals Ltd, Sydney) (100 mL) was added along with 200 mL of ethanol. The mixture was homogenised for approximately 30 seconds. The solvent extract was filtered through a Buchner funnel equipped with Whatmann #1 filter paper. The filtrate was transferred to a separating funnel and partitioned against 400 mL (4 x w/v) of 5 % hexane in petroleum ether. The non-polar layer was collected after a solvent partition and transferred to a round bottom flask. The polar layer was washed a further 2 times with 4 x w/v 5 % hexane in petroleum ether. The combined washings were dried down on an RVE. The buds were re-extracted with 4 x w/v (400 mL) of 5 % hexane in petroleum ether and sonicated for 10 minutes. The mixture was filtered through Buchner funnel equipped with Whatmann #1 filter paper. The filtrate was dried down on an RVE in a pre-weighed round bottom flask. The extracts were sub-sampled and analysed by GC FID (with derivatisation using diazomethane) and by GC FPD.

Method 2. Extraction with 0.2:1 propylene glycol to bud ratio.

Blackcurrant buds (100 g) were placed into a large porcelain mortar. Propylene glycol (20 mL) was added and the buds were gently moved around with the pestle to 'massage' the buds with propylene glycol. Once the buds were thoroughly coated, they were placed in blender with 280 mL of ethanol. The buds were homogenised in a blender for approximately 30 seconds. The solvent extract was filtered through Buchner funnel equipped with Whatmann #1 filter paper. The filtrate was transferred to a separating funnel and partitioned against 400 mL (4 x w/v) of 5 % hexane in petroleum ether. The non-polar layer was collected after a solvent partition and transferred to a round bottom flask. The polar layer was washed a further 2 times with 4 x w/v 5 % hexane in petroleum ether. The combined washings were dried down on an RVE. The buds were re-extracted with 4 x w/v (400 mL) of 5 % hexane in petroleum ether and sonicated for 10 minutes. The mixture was filtered through Buchner funnel equipped with Whatmann #1 filter paper. The filtrate was dried down on an RVE in a pre-weighed round bottom flask. The extracts were sub-sampled and analysed by GC FID (with derivatisation using diazomethane) and by GC FPD.

2.1.7. The Inclusion of Antioxidant to Improve Blackcurrant Oil Quality.

The loss of volatiles by oxidation during the extraction process may be slowed by the inclusion of an antioxidant. To test this hypothesis butylated hydroxy anisole (BHA) (Fluka Chemika), an antioxidant commonly used in essential oils at a rate of 0.02 %, was included in the extracting solvent in an attempt to reduce loss of volatiles. This extraction was conducted in duplicate alongside an identical extraction but excluding the BHA. In previous experiments (section 2.1.4. method 2), ethyl acetate was included in the extracting solvent but this resulted in low yield of volatiles. However, this may have been due to the ethyl acetate modifying the polarity of the ethanol/bud extract, enhancing the solubility of volatiles in the polar solvent. Keeping in mind the recommendations for the use of ethyl acetate in blackcurrant extraction from colleagues involved in the European industry (pers. comm. R. C. Menary), the final back extraction of the buds was altered by introducing 10 % ethyl acetate to the 5 % hexane in petroleum ether. Cursory experiments also indicated that the increased polarity resulted in improved yields (results not reported). To determine the long term effects of the inclusion of BHA all samples were analysed 26 days after the day of extraction and then again 71 days post extraction.

Method 1. Extraction with antioxidant.

Blackcurrant buds (100 g) were weighed into a blender and 100 mg of BHA was added. Ethanol (300 mL) was added and the mix was homogenised for 30 seconds. To the polar extract was added 400 mL of 5 % hexane in petroleum ether and after homogenisation the mixture was filtered through a Whatmann #1 filter paper on a Buchner funnel. The partition was allowed to separate and the non-polar layer was transferred into a round bottom flask. The polar layer was extracted a further 2 times with 400 mL of 5 % hexane in petroleum ether and the combined washes were dried down on the RVE at 40°C with a final time of 5 minutes at 60°C. The filtered buds were back extracted with 400 mL of 10 % ethyl acetate in 5 % hexane in petroleum ether with a 10 minute sonication. The extract was filtered through Whatmann #1 filter paper using a Buchner and dried down on a RVE. The samples of both the extract and the marc extract were analysed by GC FID (with derivatisation using diazomethane) and by GC FPD.

Method 2. Extraction without antioxidant.

An identical extraction was undertaken without BHA. The samples of both the extract and the marc extract were analysed by GC FID (with derivatisation using diazomethane) and by GC FPD.

2.1.8. The Storage of Frozen Buds in Ethanol to Improve Extract Yield and Quality

The loss of thiols and other quality components during the storage of frozen buds is detailed in section 3.2.2. As alcohol retards enzymatic activity, steeping the buds in ethanol prior to freezing may serve to protect some of the quality components. An experiment was conducted whereby un-rolled and rolled buds were steeped in ethanol and frozen for a period of 2 months and compared to buds frozen un-rolled and without solvent.

In the extraction process the volumes were increased to 4 x v/w ethanol is to bud weight extract to ensure effective extraction. Following extraction this volume was reduced by 50 % using RVE, to increase the concentration of components and facilitate increased recovery of volatiles into the non-polar solvent in the subsequent partition. Preliminary experiments indicated that the phase separation using this method was indistinct. It was determined that the addition of 1.4 x v/w water to bud weight allowed for a distinctive partition.

Six quantities (50 g) of commercially produced (clonal high thiol stock) blackcurrant buds were weighed into heavy-duty plastic bags. Three were frozen immediately whilst 100 mL of ethanol was added to the remaining three prior to freezing. A further 3 x 50 g of buds were rolled prior to being immersed in 100 mL of ethanol in plastic bags and frozen. After a period of 2 months the buds were transferred to flasks. Ethanol (100 mL) was added to each sample which had been frozen in 100 mL of ethanol. The buds that had been frozen without solvent were rolled and immersed in 200 mL of ethanol. All samples were ultra-turrexed and filtered through a Buchner funnel with Whatmann #1 filter paper. The filtrate was reduced by 50 % volume on RVE and 70 mL of water added to each. This was partitioned against 200 mL of 5 % hexane in petroleum ether three times. The non-polar layers were combined and dried down on the RVE. The remaining solids were extracted with 200 mL of 10 % ethyl

acetate in 5 % hexane in petroleum ether and sonicated for 10 minutes. The extracting solvent was filtered and the extract dried down under RVE. The non-polar extract and the marc extracts were combined and dried down for 5 minutes at 60°C.

2.1.9. Adaptation of the New Blackcurrant Extraction Method to Commercial Operations

The extraction technique developed through the preceding sections had been developed in the laboratory and often the practical limitations in transferring the techniques to large industrial scale processes are not always evident. After consultation with industry it was determined that the direct transfer of the new methodology to commercial operations was not feasible and the non-viable aspects were subject to further experimentation. The volumes of ethanol were too high to be easily managed in the large-scale extracting drum at the factory, not with standing the increased expense of the solvent. Indeed it was considered favourable if the volumes of non-polar solvent could also be reduced. The transfer of the ethanol extract to allow for the reduction in volumes was also problematic as was the addition of water, which precluded the recycling of solvent. Exploratory trials were undertaken to obtain basic overviews of the effect of altering a range of parameters. The treatments were not conducted in duplicate until a basic framework for an industrial based extraction methodology was established. The aspects considered un-viable by industry and investigated in the following chapters were

- could the volumes of ethanol be reduced
- exclusion of the drydown by RVE
- omission of the addition of water
- reduction in non-polar solvent volumes

2.1.9.i. The Effect of Reduced Volumes of Ethanol on Yields and the Subsequent Limitations on Recycling Solvent.

The first step of the method developed in the laboratory required a 4 : 1 volume : bud ratio of ethanol. In the industrial context the volume of ethanol was limited by the capacity of the main extraction vessel. Buds are extracted in lots of 400 kg, requiring

1600 L of ethanol. In addition the increase in ethanol use would increase production costs, though this may be offset by recycling the solvent. Minimising the amount of ethanol used in the first extraction was one of the main priorities. As such the recoveries of extract and the amount of ethanol recovered from sequential extractions of blackcurrants were investigated.

Frozen blackcurrant buds (50 g) from a commercial harvest were rolled and immediately covered with 100 mLs of ethanol (2 : 1). The buds were transferred to polypropylene bags and placed in a stomacher for 2 hours. There appeared to be adequate solvent during this process to keep all the buds covered. After extraction the buds were filtered through a Buchner funnel equipped with Whatman #1 filter paper and the filtrate was dried down on the RVE at 30 - 40°C. The buds were returned to the stomacher bag, extracted in a further 50 mL of ethanol (1 : 1), filtered and the filtrate dried down by RVE. This step was repeated a further 2 times and the weights of the extracts produced at each extraction were recorded.

2.1.9.ii. Exclusion of the Volume Reduction Step During Ethanol Extraction and the Effect of the Addition of Water on the Partitioning Between Polar and Non-polar Layers.

The reduction in volume of the ethanolic extract undertaken in the laboratory was not easily transferable to the industry context. It would be expedient to exclude this step in the extraction process. This experiment was undertaken to determine whether a distinct partition was still evident when the volume of the ethanol extract was not reduced using RVE.

Frozen blackcurrant buds (50 g) were rolled and immediately immersed in 2 : 1 ethanol (100 mL) and ultraturrexed until the buds were finely blended. The mix was filtered through a Buchner funnel equipped with Whatmann filter paper #1 under vacuum. A further 50 mL of ethanol was added to the buds and the mix was placed in an ultrasonic bath for 5 minutes. The mix was filtered and the filtrates combined. The solvent recovered from the 100 and 50 mL washes were 69 and 65 mL respectively, giving a total solvent recovery of 89 %. The combined filtrate was divided into two 67 mL aliquots to allow for two experiments to determine the effect of the addition of

water to the ethanol extract prior to the partition of extracted components into non-polar solvents.

Method 1. Extraction with the addition of water.

In the original laboratory method the amount of water added relative to the buds was a 1.4 : 1 (v/w) ratio. Distilled water (35 mL) was added to 67 mL of the filtrate and a further 12.5 mL of ethanol was added. A reduction from 4 to 3 washes of the polar layer with 5 % hexane in petroleum ether at a ratio of 1.5 : 1 (w/v) would also reduce the cost of extractions. As such the ethanol extract was washed 3 times with 38 mL of 5 % hexane in petroleum ether. The partition effected was slow to separate and indistinct. As a result this part of the experiment was halted.

Method 2. Extraction without water.

The second aliquot of 67 mL of ethanol filtrate was partitioned into 3 x 38 mL of 5 % hexane in petroleum ether without the volume reduction and excluding water. The extracts were dried down on the RVE at 40°C.

2.1.9.iii. Inter-comparison of the Laboratory Based Extraction Method to the Method Modified to Meet the Limitations of the Industrial Scale Extraction.

Based on the outcomes detailed in section 3.1.9 a blackcurrant bud extraction method modified for industry was advanced. In this experiment the extract produced from the modified protocol was compared to the original laboratory based method. Previously all experiments had employed laboratory based equipment to agitate buds in extracting solvent. This had been adequate to inter-relate yields for laboratory based extractions. However methods were required to more closely mimic those implemented in the factory. The agitator used in industry was a rotating cylindrical stainless steel drum of volume 5.7 m^3. The closest apparatus available in the laboratory was a soil extractor comprising of a large number of glass bottles fixed to a rotating drum. All extractions undertaken to assess the viability of modifications to industry used the soil extractor described.

Method 1. The original laboratory developed method.

Recently harvested commercially produced White Bud buds (100 g) were rolled frozen. The buds were immersed 400 mLs of ethanol. The bottles were sealed with rubber bungs and agitated on a rotary drum. It was evident that the ethanol volume was too high with the small air void in the glass vessels effectively cushioning the impact of the buds as the drum rotated. The buds were ultraturrexed for 5 minutes and then returned to the rotating drum for a further 2 hours. The solution was decanted through a Buchner funnel using Whatmann #1 filter paper and the solution was reduced in volume by 50 % using RVE. Water (140 mLs) was added to the solution and partitioned into 400 mLs of 5 % hexane in petroleum ether. The extraction of the ethanol layer was repeated a further 2 times and the non-polar layers were combined and dried down by RVE. The remaining buds were sonicated for 10 minutes in 400 mLs of 10 % ethyl acetate in 5 % hexane in petroleum ether. The solvent was decanted and filtered using a Buchner with Whatmann #1 filter paper and dried down by RVE. GC samples were taken at each step to ascertain the effectiveness of extraction.

Method 2. Extraction process modified for industry.

Recently harvested commercial White Bud buds (100 g) were rolled frozen. The buds were placed in bottles and 200 mLs of ethanol was added. The bottles were sealed with rubber bungs and agitated on a rotary drum for 2 hours. The solution was decanted through a Buchner funnel using Whatmann #1 filter paper. Another 100 mLs of ethanol was added to the buds and the bottles again stoppered and rotated for 30 minutes. This was also filtered and the filtrates combined. In a separating funnel the combined filtrates were partitioned 3 times with 75 mLs of 5 % hexane in petroleum ether. The buds were back extracted with 100 mLs of 10 % ethyl acetate in 5 % hexane in petroleum ether for 30 minutes on the rotating drum. This was repeated twice and the marc extracts combined with the combined non-polar fractions from the partitioning of the ethanol extract. GC samples were taken at each step to ascertain the effectiveness of each step.

2.1.9.iv. Continued Development of the Industry Based Extraction Method – effect of ultra-turrexing and the importance of the volume reduction and inclusion of water in the polar extract.

The results from section 2.1.9.iii. could not be related directly as the buds in the laboratory method were ultra-turrexed as well as extracted on the rotary drum. This was because incomplete extraction occurred in the laboratory trials as a result of excess volume of buds in the extracting vessel. To determine whether the inclusion of an ultra-turrexing step had significantly changed the extraction efficiency the modified method of extraction was repeated and a second extraction was undertaken identical to the modified method but with the inclusion of 5 minutes of ultra-turrexing. In addition each extracting method was divided into a further 2 experiments to revisit the importance of the reduction in volume of the ethanol extract and the addition of water to effect a clean partition.

Method 1. Extraction using an ultra-turrex.

Recently harvested commercial White Bud buds (100 g) were rolled frozen, placed in bottles and 200 mLs of ethanol was added. The buds were ultra-turrexed for 5 minutes. The bottles were sealed with rubber bungs and agitated on a rotary drum for 2 hours. The solution was decantedand filtered through Whatmann #1 filter paper. Another 100 mLs of ethanol was added to the buds and the bottles again stoppered and rotated for 30 minutes. This was also filtered and the filtrates combined. The 236 mLs of ethanol extract was divided equally between two experiments

Method 1-a. The extract (118 mLs) was reduced by 50 % by RVE and 25 mLs of water was added. The extract was placed in a separating funnel and partitioned 3 times with 0.75 : 1 of 5 % hexane in petroleum ether. The buds were back extracted with 1 : 1 10 % ethyl acetate in 5 % hexane in petroleum ether with 30 minutes on the rotating drum. This was repeated twice and the marc extracts combined with the non-polar fractions from the partitioning of the ethanol extract.

Method 1-b. The extract (118 mLs) was not reduced in volume and had no water added prior to partitioning against the non-polar solvent. The extract was placed in a separating funnel and partitioned 3 times with 0.75 : 1 of 5 % hexane in petroleum

ether. The buds were back extracted with 1 : 1 of 10 % ethyl acetate in 5 % hexane in petroleum ether for 30 minutes on the rotating drum. This was repeated twice and the marc extracts combined with the combined non-polar fractions from the partitioning of the ethanol extract.

Method 2. Extraction not using an ultra-turrex.

Method 1 was repeated without subjecting the buds to 5 minutes of ultra-turrexing. The ethanol extract recovery was 242 mLs. The ethanol fraction was divided equally between two experiments.

Method 2-a. The extract (121 mLs) was reduced by 50 % by RVE and 25 mLs of water was added. The extract was placed in a separating funnel and partitioned 3 times with 0.75 : 1 of 5 % hexane in petroleum ether. The buds were back extracted with 1 : 1 10 % ethyl acetate in 5 % hexane in petroleum ether with 30 minutes on the rotating drum. This was repeated twice and the marc extracts combined with the non-polar fractions from the partitioning of the ethanol extract.

Method 2-b. The extract (121 mLs) was not reduced in volume and had no water added prior to partitioning against the non-polar solvent. The extract was placed in a separating funnel and partitioned 3 times with 0.75 : 1 of 5 % hexane in petroleum ether. The buds were back extracted with 1 : 1 of 10 % ethyl acetate in 5 % hexane in petroleum ether for 30 minutes on the rotating drum. This was repeated twice and the marc extracts combined with the combined non-polar fractions from the partitioning of the ethanol extract.

2.1.10. Stability of Endogenous Thiols in Blackcurrant Extracts

In previous experiments (section 2.1.7) antioxidants were included in the extracting solvent to retard any possible depletion of volatiles throughout the extraction process. The loss of the thiol from the extract once blackcurrant extract is produced is a separate issue. Re-analysis of commercially produced blackcurrant extracts identified the loss of the thiol over a period of months during storage at <5°C as detected by GC FPD (results not reported). Experiments were established to firstly determine the rate of depletion of the endogenous thiol and secondly to assess the effectiveness of antioxidants to retard depletion if it is occurring.

2.1.10.i. The Rate of Depletion of Thiols in Commercially Produced Blackcurrant Extracts.

Three blackcurrant extracts produced by industry were sub-sampled (described section 2.1.v) in duplicate and analysed by GC FPD using the conditions described in section 2.1.iii. Samples were stored at 4°C over a period of a month and sub-sampled periodically to establish the rate of dissipation of 4-methoxy-2-methyl-2-butanethiol in blackcurrant concrete.

2.1.10.ii. Effect of Extraction Protocols and Antioxidants on the Depletion of Thiols in Blackcurrant Extracts.

The experiments detailed previously in 2.1.7 investigated the inclusion of antioxidants in the extracting solvent with view to protecting the blackcurrant labile components during the extraction process. In addition the buds were steeped in ethanol to retard enzymatic acitivity that may have contributed to the loss of quality components. The potential for the addition of antioxidant to the final product and the potential for ethanol based extractions to protect labile species in the final product were investigated. Humeticants, (a substance that absorbs water) such as propylene glycol, have also been reported to have an anti-oxidant effect by altering the availability of water to degradative processes. Experimentation was instigated to determine the rates of depletion in;

1. extract produced using the standard methods (5 % hexane in petroleum ether)
2. extracts fortified with humeticant, (standard 5 % hexane in petroleum ether)
3. extracts fortified with antioxidant (standard 5 % hexane in petroleum ether)
4. extracts produced using an ethanol based extraction methodology
5. extracts fortified with humeticant (ethanol based extraction methodology)

Samples from all four methods were analysed by GC FID and GC FPD on the day of extraction and at 1, 5, 7, 14, 21, 75 and 92 days.

<u>Method 1. Standard method (5 % hexane in petroleum ether)</u>

Rolled buds (262 g) were divided between 2 glass bottles. Hexane (5 %) in petroleum ether was added at a ratio of 2.5 : 1 relative to bud weight. The bottles were sealed with stoppers and placed on the rotating drum for 3 hours. The solvent was decanted

off and a further 2.5 : 1 solvent added. After rotation for a further 3 hours a third and final extraction of 1.25 : 1 of 5 % hexane in petroleum ether was conducted with 2 hours of rotation. The extracts were combined and dried down by RVE at 40°C.

Method 2. Standard method (5 % hexane in petroleum ether) with humeticant.
The procedures followed in extraction method 1 were repeated, however after the final dry down 10 % propylene glycol was added based on an expected 3 % extract yield (0.3 mL / 100g of blackcurrant bud extracted).

Method 3. Standard method (5 % hexane in petroleum ether) with BHA.
The processes detailed in extraction method 1 were replicated, however, butylated hydroxy anisole (BHA) was added to extracts prior to dry down. The amount added was calculated based on the projected yield of 3 % of concrete from the 130 g bud extraction. BHA (0.78 mg) was added to each of 2 x 130 g blackcurrant bud extracts prior to dry down. This amounted to 0.02 % BHA content based on extract weight.

Method 4. Modified ethanol extraction method.
Rolled blackcurrant buds (400 g) were divided between 4 glass bottles. Ethanol was added at a 2:1 ratio relative to bud weight. The bottles were rotated for 2 hours. The ethanol was decanted and a further 1:1 ethanol added and the buds rotated for a further 30 minutes. The ethanol extracts were combined and 300 mL of 5 % hexane in petroleum ether was added to the solvent in a separating funnel. After mixing thoroughly a partition was allowed to form and the non-polar layer was separated. The partition step was repeated twice (0.75:1). The 4 x 100 g extracted buds were each further extracted in 100 mL of 10 % ethyl acetate in 5 % hexane in petroleum ether. The bottles were again rotated for 30 minutes and the solvent removed. This was repeated twice. The non-polar extracts were combined and the samples dried down.

Method 5. Modified ethanol extraction method with humeticant.
The procedures followed in extraction method 3 were repeated, however after the final dry down 10 % propylene glycol was added based on an expected 3 % extract yield (0.3 mL / 100g of blackcurrant bud extracted).

Section 2.2. DORMANCY, FREEZING AND INCUBATION

In this section the White Bud variety was compared with a new variety cloned from plants selected from White Bud plantations that were found to have higher levels of 4-methoxy-2-methyl-2-butanethiol. This thiol contributes the catty note to blackcurrant extracts and is associated with quality products. The experiments look firstly at the how the levels of different components in both selections change through the dormant stage of the plant cycle to determine the optimum time for harvest. In commercial operations all harvest buds are frozen prior to extraction. Experiments were designed to determine if this has detrimental effects on the extracts subsequently produced. Also the potential to improve the quality of the extracts with a period of post-harvest incubation to allow for continued synthesis of important components. It was necessary to determine whether such post-harvest synthesis was retarded or enhanced by freezing prior to incubation and whether the date of harvest was also relevant to the potential to improve extract yield and quality.

2.2.1. Variation During Dormancy of the Chemical Composition of Blackcurrant Buds

In Tasmania, Australia, the main commercial blackcurrant cultivar is White Bud, which is a selection from the widely-grown European cv. Baldwin. Higher levels of 4-methoxy-2-methyl-2-butanethiol have been detected in some plants of cv. White Bud grown at the Horticultural Research Centre at the University of Tasmania. This study investigates the changes in composition of extracts of buds of cv. White Bud and the selected high thiol clones through dormancy.

Samples were collected from a plot of cv. White Bud and from six plots of high thiol clones grown at the University Research Farm in Cambridge, Tasmania (site 1). Two replicate samples were harvested from each plot at monthly intervals. At a second site, where only the cv. White Bud was cultivated for the commercial production of blackcurrant buds, five samples were collected fortnightly and extracted in duplicate.

At both sites 20 stems were collected from each plot. The buds were removed from the stems by hand, counted and weighed into beakers. Amounts of each were sub-sampled, weighed into paper bags and dried at 65°C for over 72 hours to determine

dry weight of buds. The remaining buds were then crushed under liquid nitrogen and transferred to conical flasks. The ground buds were extracted with 4 x w/v of 5 % hexane in petroleum ether. Each flask was fortified with the equivalents of 1.7 μgmL^{-1} octanethiol and 0.8 $mgmL^{-1}$ octadecane, internal standards for FPD and FID respectively. The flasks were sonicated for 10 minutes and allowed to settle for 20 minutes. Aliquots (0.5 mL) of each sample were transferred into GC vials. The terpene acids were methylated with diazomethane. Excess reagent was quenched with glacial acetic acid and the sample analysed by GC FID for volatile components and methylated acids as described in section 2.1.1.ii. A further 1 mL was sub-sampled into a second GC vial and analysed immediately by GC FPD to quantify the level of 4-methoxy-2-methyl-2-butanethiol in the buds under the conditions described in section 2.1.1.iii.

2.2.2. Effect of Freezing on the Volatile Components in Blackcurrant Buds.

In commercial operations it is necessary to freeze buds prior to extraction until sufficient quantities are accumulated to warrant a large-scale extraction. The experiment described herein investigates the effects freezing has on the volatile components in blackcurrant buds over time. Fresh buds (18 x 10 g) were weighed into 250 x 305 x 0.007 mm HDPE poly bags. Three samples were extracted immediately and the remainder frozen at -18°C. Samples were removed at intervals over a period of 6 months and thawed at room temperature. Buds were extracted in 4 x w/v 5 % hexane in petroleum ether and blended to a fine suspension using 3 x 15 second bursts of an ultra turrex. Octanethiol (16.6 mg) and octadecane (41.75 mg) were added as internal standards for GC FPD and GC FID analyses respectively. Samples (1 mL) of each solvated extract was transferred to a GC vial and analysed immediately using the conditions described in section 2.1.1.iii. A further 0.5 mL was transferred to a second GC vial in which the terpene acids were methylated with diazomethane, the excess of which was quenched with glacial acetic acid and the sample analysed by GC FID as described in section 2.1.1.ii.

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2.2.3. Effect of Incubation on the Components of Blackcurrant Buds (laboratory scale)

2.2.3.i. Incubation of Intact and Rolled Buds of the Commercial Blackcurrant cv White Bud.

Incubating buds for periods of time prior to extraction has the potential to allow for post-harvest synthesis of blackcurrant volatiles. The aspects examined are

- For what length of time does post-harvest synthesis continue?
- Are intact buds as productive as buds damaged by rolling?
- Does freezing deactivate or promote post-harvest synthesis?

The first series of incubation experiments used the cv White Bud variety (commercial). The effect of incubations of buds at 10°C over a period of 20 days in an aerobic environment was investigated. Machine-harvested buds from the commercial site were well mixed. Half of the buds were rolled. Samples of 18 x 10 g of each of the intact and rolled buds were weighed into 250 mL conical flasks and placed in a dark, controlled temperature room at 10°C. Samples were removed and extracted at periods from 0 - 500 hours (~20 days). Solvated extracts were produced as described in section 2.1.1.iv and analysed under GC conditions reported in sections 2.1.1.ii and 2.1.1.iii.

A further 18 x 10 g of each of the intact and rolled buds were weighed into 250 x 305 x 0.007 mm HDPE poly bags and frozen. After 84 days the buds were thawed at room temperature and incubated and extracted as described above for the fresh, unfrozen buds.

2.2.3.ii. Incubation of Machine Harvested and Hand-cut Buds High Thiol Clones.

The second series of experiments used high thiol clones (HTC). Incubations similar to those undertaken for the commercial White Bud varieties were conducted. However in this case two harvesting methods were used, namely machine and hand-cut incubated at 10°C, under either air or nitrogen. The aspects investigated in summary are:-

- Does the potential for post-harvest synthesis vary between the White Bud and HTC?
- Are the post-harvest synthetic processes aerobic or an-aerobic?
- Does the damage incurred during machine harvest retard or promote post-harvest synthesis when compared to buds cut by hand?

Buds were gently but thoroughly mixed and 2 x 15 of 10 g lots of each of the machine harvested and hand-cut buds were weighed into 250 x 305 x 0.007 mm HDPE poly bags. Fifteen of each of machine harvested and hand-cut buds were left open to the air whilst a further fifteen of each were flushed with nitrogen and sealed. Samples were stored in a controlled temperature room at 10°C in the dark and, at set intervals over 72 hours, were removed and frozen at -18°C prior to extraction. Extractions followed the procedures referred to in the previous section.

2.2.4. Pilot-scale Incubation of Blackcurrant Buds for Improved Extract Quality and Yield

The results from section 2.2.3 presented in section 3.2.3 indicated that the yield of important volatiles from blackcurrant buds may be improved by post-harvest incubation. However, only solvated extracts were produced such that yields could only be extrapolated for GC amenable components with calculations based on a 1 : 1 GC FID response ratio relative to the internal standard, octadecane. In addition full aroma assessments could not be undertaken. When results for all experiments were combined the level of volatiles in buds during dormancy and the effects of incubation (section 2.2.1) suggested a large-scale trail be established to investigate:-

- the optimal time of bud harvest to maximise volatile yield.
- the effectiveness of incubation of buds at each harvest date.
- the impact of harvest date and incubation on aroma.

A uniform section of a commercial blackcurrant plantation in Southern Tasmania was divided into 4 blocks with 5 times of harvest as treatments. Each treament (50 m of row) was sufficiently large to allow 1.2 kg of buds to be harvested at each treatment

time. The canes were machine harvested and the buds were taken to the laboratory and frozen for 2 weeks. The buds were divided into 3 x 300 g sub-samples from each block. The first 300 g sample from each block was removed from the freezer after 2 weeks and extracted immediately. The second replicate from each block was removed from the freezer after 2 weeks and left to incubate at 10°C for 72 hours prior to extraction. The third set of replicates were thawed after 2 weeks and incubated at 10°C for 8 days prior to extraction. The extraction method was based on the standard protocols as described below

The trial was conducted in the season of 2001 and repeated again in 2002. Due to a communication problem the canes to be collected at the fourth harvest date in the 2002 season was mulched by the farmer. In addition the samples from the July 2001 harvest that had been incubated for 72 and 190 hours were extracted in re-cycled solvent. The error was identified when the final extracts were low in viscosity. The samples were re-dissolved in 60 mLs of ethanol and dried down at 40°C with a final dry down on the RVE at 45°C for 2 minutes. The yield of oils was high increasing by up to 20 % for the oils incubated for 72 and 190 hours relative to the non-incubated oils produced in the previous month. However in 2002 fresh solvent was used and similar marked increased occurred as recorded in 2001.

Extraction method

Buds were rolled immediately prior to extraction. Each replicate was divided into 500 mL bottles and 5 % hexane in petroleum ether was added in a ratio of 2.5 : 1 relative to bud weight. The bottles were stoppered and placed on a rotating drum for 3 hours then the solvent was decanted. Another 2.5 : 1 ratio of solvent was added and the buds rotated for a further 3 hours then left to soak overnight. The solvent was again decanted and a final wash of 2.5 : 1 was rotated for 2 hours, decanted and the 3 washes combined. The solvent was dried down at 40°C with a final 5 minutes at 60°C. All samples were analysed by GC FID and GC FPD (sections 2.1.1.ii and 2.1.1.iii). All samples were also subjected to aroma assessment as described below.

Aroma Assessment

Fifteen people were tested for their ability to distinguish and evaluate blackcurrant extracts by subjecting them to a triangle test as detailed in the publication 'Laboratory Methods for Sensory Evaluation of Food' (Lammond, 1977). Extracts were dissolved in ethanol (1 % w/v) added dropwise to 100 mLs of distilled water in wine glasses and left for 30 to 60 seconds prior to sniffing. Participants were required to distinguish between an extract produced commercially by the local blackcurrant industry and an extract regarded as of high quality by international markets and shown to be high in 4-methoxy-2-methyl-2-butanethiol. Ten of the participants were successful in distinguish between the extracts and were deemed qualified to contribute as judges to determine the quality of the blackcurrant extracts produced in the pilot-scale harvest and incubation trials.

The twelve samples produced from the trial of 4 harvest dates and 3 incubation periods were dissolved in ethanol at a 1% w/v ratio. Judges were required to evaluate the degree of cattiness relative to a commercial sample produced using similar extraction techniques and from similar bud varieties. A second reference, deemed of high quality with high levels of the 4-methoxy-2-methyl-2-butanethiol was made available for participants to re-establish the nuances of the catty note. Each sample from the trial was assessed for the degree of cattiness. Judges had first to determine whether the cattiness in the extract in question was of higher, equal or lower potency than the reference commercial extract. The degree of difference was then placed in one of five categories, no difference, slight, moderate, high or of extreme difference. Each possible result was assigned a score between 1 and 9, 1 being for samples lower in cattiness than the reference sample to an extreme degree whilst 9 was assigned to samples higher in cattiness than the reference sample by an extreme degree:

low cattiness		<		reference sample			<	high cattiness
extreme	high	moderate	slight	no difference	slight	moderate	high	extreme
1	2	3	4	5	6	7	8	9

The evaluation was repeated for the samples produced in the harvest year of 2002. As there were only three harvest dates, however, only 9 samples were available for quality assessment.

2.2.5. Commercial-scale Incubation of Blackcurrant Buds for Improved Extract Quality and Yield

It has been demonstrated on the laboratory and pilot-scale that the levels of endogenous thiols in blackcurrant extracts can be increased by holding the buds at ~ 10°C for 72 hours. Two small pilot experiments were also conducted in the 2002 harvest. In commercial operations the machine-harvested buds are collected from the outlet of the harvester and placed into lidded 60 L plastic lined cardboard boxes that can hold approximately 10 kg of blackcurrant buds. Boxes are placed in a -15°C freezer until sufficient quantities have been accumulated to warrant a large-scale extraction. In the first experiment instead of placing the buds immediately into the freezer, several boxes of buds were spread on the factory floor in small piles at ambient temperature. A sample was taken immediately and frozen, whilst a further 2 samples were taken at 24 and 72 hours. As this experiment was not replicated the experiment is not detailed in this thesis. However analyses confirmed an increase in thiol levels after 24 hours but this was followed by a decrease in the sample taken at 72 hours. The second experiment was conducted with 4 boxes of fresh buds. Box lids were left ajar and the plastic box liners were left loosely closed over the buds. The boxes were left on the floor of the factory and sample of 120 g of buds were taken immediately and at 24 and 72 hours. Samples were frozen for 2 weeks prior to extraction. Thiols increased in this instance by 25 % after 72 hours. The % oil yields on a fresh weight basis over the period was 3.01, 3.06 and 2.98 at 0, 24 and 72 hours respectively. The results from all these experiments suggested that incubation trials should be tested on a commercial scale.

For each bulk extraction 4 replicates of the 2 treatments:–

1. Freezing immediately on arrival at the factory
2. Incubation at room temperature for 72 hours prior to freezing.

A batch extraction undertaken by industry is usually ~400 kg. The mechanical harvester moves between plantations producing approximately 10 boxes of buds daily. To accumulate sufficient material to justify a 400 kg extraction, buds from several farms are usually combined. The degree of variation in buds sourced from different growers, areas of cultivation and variety was expected to be high. However, four replicates of each treatment were required. To overcome the variability the 10 boxes normally harvested on a daily basis was divided into 2 lots of 5 boxes. One lot was to be incubated whilst the second lot was placed directly in the freezer. Attention was taken to try to ensure that boxes sourced from a particular grower or plantation was represented in each lot. That is, for example, if two boxes from one plantation arrived at the factory, one went to the incubation treatment prior to being frozen and the other went directly to the freezer. Each of the 2 sets of 5 boxes were labelled and placed in separate sections of the freezer. When sufficient quantities of each had been collected batch extractions were undertaken ensuring each treatment were extracted separately. Extractions of the non-incubated buds were interspersed between extractions of the incubated buds. The method of extraction undertaken was the standard 5 % hexane in petroleum ether.

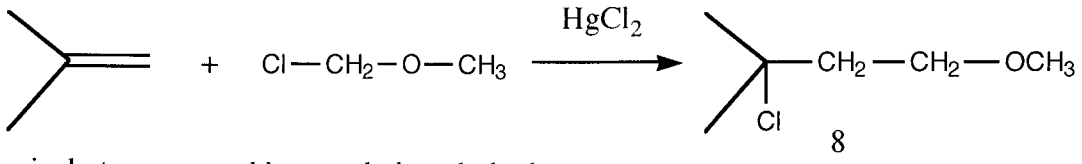
Section 2.3. SYNTHESIS OF THIOLS

An empirical approach to the study of harvest and extraction technology by adjusting extracting solvents and protocols to optimize quality and yield is effective in producing results that may be immediately adopted by industry as shown in sections 2.1 and 2.2 and reported in sections 3.1 and 3.2. However, understanding the underlining biosynthetic processes may provide for more direct and accurate determination of the physical parameters that regulate the chemical profile of natural extracts. Accessibility to the chemicals that confer a particular quality allows for direct assessment of chemical lability and thresholds at which their contribution is effective in the aroma profiles. Identifying the precursors to the important components provides the opportunity to investigate the conditions such as nutrition and stage of dormancy that result in higher levels of biosynthesis. In the following sections the synthesis of 4-methoxy-2-methyl-2-butanethiol is undertaken. A possible precursor to this important component based on the chemistry reported in relevant literature for similar odour active chemicals is proposed. A successful production and identification of the thiol precursor in blackcurrant buds would allow for preliminary trials to investigate the levels maintained through dormancy relative to the concomitant levels of the thiol.

2.3.1. Synthesis of 4-Methoxy-2-methyl-2-butanethiol

Riguad *et al.*, (1986) published a two stage synthesis of 4-methoxy-2-methyl-2-butanethiol (1). In this study the access to the thiol by direct synthesis would allow for the establishment of more accurate standard curves and provide for experiments on the effect of temperature and solvent composition on the stability of the chemical. The aroma detection thresholds of the thiol may also be investigated as well as possible synergistic effects that may be occurring with other components of blackcurrant extracts. As such the synthesis reported by Riguard *et al.*, (1986) was undertaken. The two stage synthesis is presented in scheme 1 and 2.

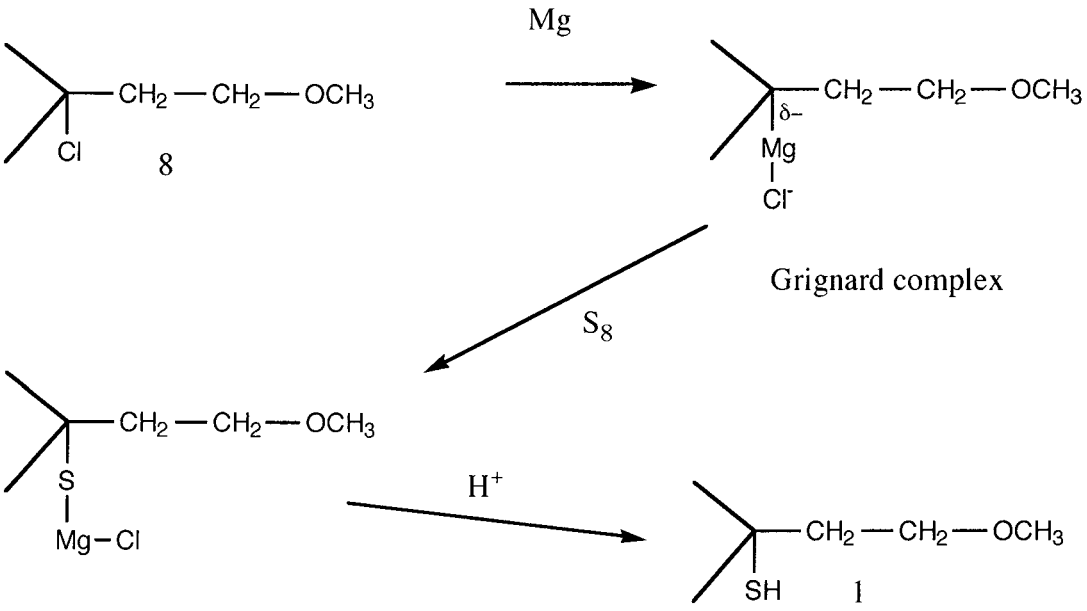
Stage 1.



isobutene chloromethyl methyl ether 2-chloro 4-methoxy 2-methyl butan

Scheme 1.

Stage 2



4-methoxy-2-methyl-2-butanethiol

Scheme 2.

Stage 1. Synthesis of 2-chloro-4-methoxy-2-methylbutane (8)

Chloromethyl methyl ether (supplied by Sigma Aldrich, St Louis, USA) (80.51 g, 1.4 mole) and mercuric chloride (4g) (Fluka Chemika) were weighed into the glass lining of an hermetically sealable reaction vessel. Isobutene (Sigma Aldrich) (67.2 g, 1.2 moles) was introduced into the chamber through a gas intake valve. The mixture was agitated for 1 hour and then left for two days. The solution was transferred to a round bottom flask then distilled under a vacuum of 50 mm Hg. Four fractions were collected. All fractions were analysed by benchtop GC mass selective detector (MSD) as described below. Fraction 2 (50°C - 65°C) contained the target product, 2-chloro-4-

methoxy-2-methylbutane (VIII). The recovery of the chlorinated butane in fraction 2 was 10.5 %. This was based on a n estimated purity of 70% as determined by peak areas of the chomatogram acquired by GC MS.

Analyses by GC MSD

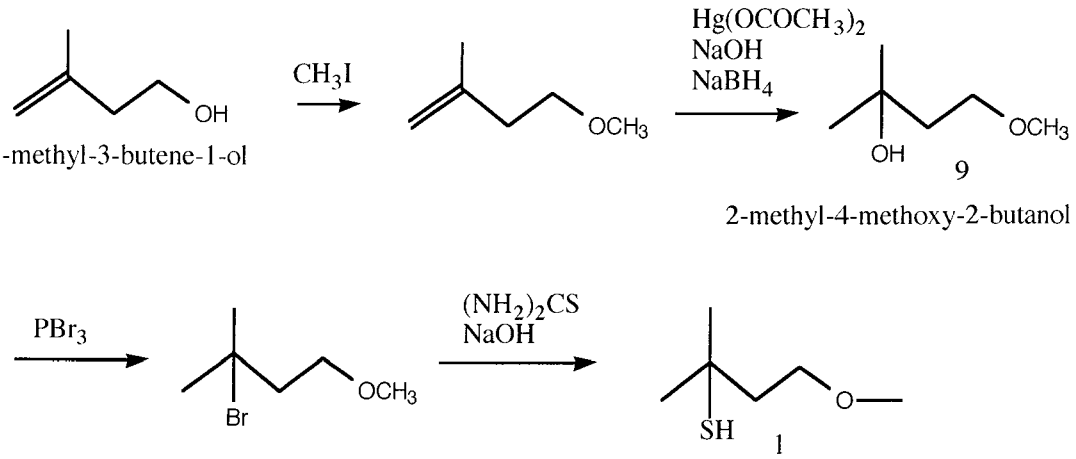
Samples were analysed using a Varian 3800 GC coupled to a Varian 1200 triple quadrupole mass spectrometer. The GC was equipped with a Varian Factor Four VF-5ms column (25 m x 0.25 mm id, 0.25 μm film thickness). Automatic injections of 1μL with a split ratio of 20:1 were applied to the column that was supplied with helium at a flow rate of 1.2 $mLmin^{-1}$. The GC oven was held at 40°C for 3 minutes followed by a temperature gradient of 20°$Cmin^{-1}$ to 170°C. The ion source temperature was 200°C and the mass spectrometer (Q1) scanned from the m/z of 35 to 300 every 0.3 seconds.

Stage 2. Synthesis of 4-methoxy-2-methyl-2-butanethiol

All glassware was placed in an oven overnight at 60°C. The reagents, magnesium (Riedel-de Haën) and sulfur (Riedel-de Haën), were placed in a dessicator overnight. Nitrogen was flushed through a three necked flask equipped with a condenser and placed on a heated magnetic stirrer. Magnesium (0.4 g) was placed in the flask and anhydrous ether (2 mL) was added. The system was kept under a low pressure nitrogen atmosphere. The condenser was carefully lifted and methyl iodide (Aldrich) (45 μL) was added. Bubbling was immediately evident. Anhydrous ether (4 mL) was first drawn into a syringe followed by 2-chloro-2-methyl-4-methoxybutane (1 mL). The syringe was inverted several times to ensure dissolution into the ether and the solution was added drop-wise through a septum on one of the flask necks. Bubbling was immediately evident and there were signs of reflux at the base of the condenser. The remaining 2-chloro-2-methyl-4-methoxybutane (total 0.14 moles) was added over a period of 30 minutes. The sulphur (6.4 g, 0.2 moles) was added and bubbling continued for over an hour. The reaction was quenched with water and then dilute sulphuric acid. The ether fraction was analysed by GC FPD. There was no evidence of the target synthetic thiol.

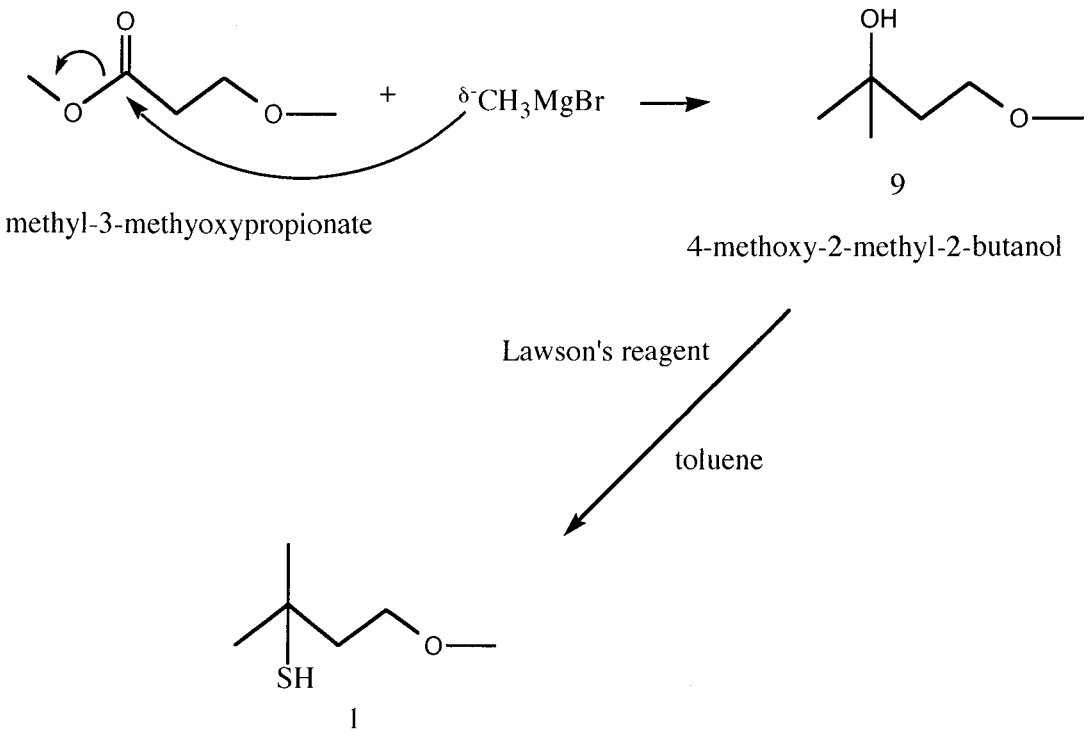
2.3.2. A Novel Synthesis of 4-Methoxy 2-methyl-2-butanethiol

Despite repeated attempts using the procedures detailed in section 2.3.1 4-methoxy-2-methyl-2-butanethiol was not successfully synthesised. In addition the reagent chloromethyl methyl ether is a listed carcinogen and is only available from the United States by sea freight, a clearance process which sometimes takes more than a year to complete. Research into the odorants of virgin olive oils (Guth and Grosch, 1991) resulted in the development of a new synthetic path to 4-methoxy-2-methyl-2-butanethiol in a 4 stage process using 1-methoxy-3-methyl-3-butene as the starting product (scheme 3).



Scheme 3.

However a more direct route to the intermediate 4-methyl-2-methyl-2-butanol (9), and the subsequent thiolation of the alcohol group was devised. Methyl-3-methoxypropionate is a readily available and inexpensive reagent. When reacted with a Grignard reagent the ester is converted to the intermediate ketone which then reacts further to a tertiary alcohol. In a second step this alcohol may then be converted into a thiol using Lawesson's reagent (scheme 4).



Scheme 4.

Stage 1. Synthesis of 4-methoxy-2-methyl-2-butanol (9)

A 3 M diethyl ether solution of methyl magnesium bromide (Sigma Aldrich, St Louis, USA) (6 mL, 18 mmoles) was dispensed in a three necked reaction vessel that had been flushed with nitrogen. Methyl-3-propionate (Sigma Aldrich) (1 mL, 8.4 mmoles) was added drop-wise. The subsequent reaction was vigorous. The reaction was quenched with sulphuric acid (10 mL of 1 N) and the mixture was washed with diethyl ether and decanted into an Erlenmeyer flask. The aqueous solution was then washed with diethyl ether (2 x 2.5 mL) and water was added until the white amorphous precipitate dissolved. The solution was distilled under vacuum (50 mm Hg) and a distillate (0.58 g) was collected at 100 to 104°C.

Stage 2. Synthesis of 4-methoxy-2-methyl-2-butanethiol (1)

Lawesson's reagent (LR: 2,4-bis(p-methoxyphenyl)-1,3,2,4-dithaphosphetane 2,4-disuphide) is a commonly used reagent for the synthesis of sulphur containing compounds. Researchers at the University of Tsukuba in Japan (Nishio, 1989)

reported the reaction of the (1,2)-N-acylamino secondary alcohol with an equimolar amount of Lawesson's reagent in toluene at reflux temperature under argon for 30 minutes yielding 2,5-diphenylthiazoline in a 56 % yield. This method was applied to the thiolation of the secondary alcohol 4-methoxy-2-methyl-2-butanol.

Lawesson's reagent (Sigma Aldrich) (17.23 g) was placed in a round bottom flask and toluene (20 mL) was added followed by 4-methoxy-2-methyl-2-butanol (9) (5 mL, 42 mmoles) and the solution kept at room temperature for 2 hours. The solution was decanted into a round bottom flask and distilled. Distillation commenced at less than 35°C but eventually rose to 66°C before decreasing to 40°C. Toluene has a boiling point of 110.6°C.

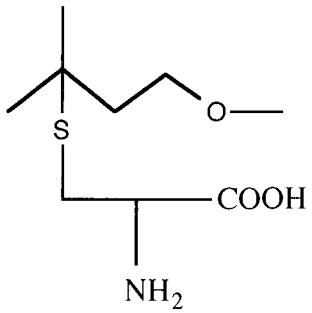
Analysis by GC mass selective detection (MSD) using conditions detailed below, confirmed the successful synthesis of 4-methoxy-2-methyl-2-butanethiol (1). However the solvent toluene had co-distilled with the thiol as shown in the chromatogram displayed in figure 3.3.vi. The mass spectrum (MS) of the synthetic thiol is also shown.

Analyses by GC MSD

Samples were analysed using Varian 3800 GC coupled to a Varian 1200 triple quadrupole mass spectrometer. The GC was equipped with a Varian Factor Four VF-5ms column (25 m x 0.25 mm id, 0.25 μm film thickness). Automatic injections of 1μL with a split ratio of 20:1 were applied to the column that was supplied with helium at a flow rate of 1.2 $mLmin^{-1}$. The GC oven was held at 60°C for 3 minutes followed by a temperature gradient of 15°$Cmin^{-1}$ to 200°C. The ion source temperature was 200°C and the mass spectrometer (Q1) scanned from the m/z of 35 to 300 every 0.3 seconds.

Section 2.4. SYNTHESIS OF THE CYSTEINE-THIOL CONJUGATE AND MONITORING OF THE THIOL PRECURSOR IN BLACKCURRANT BUDS AND EXTRACTS.

Publications in the areas of wine production have identified cysteine conjugates as precursors to potent volatile thiols with chemical structures similar to 4-methoxy-2-methyl-2-butanethiol (Darriet *et al.*, 1993; Tominaga *et al.*, 1998a). If indeed the chemistry elucidated through research in viticulture is similar to that found in the biochemistry of blackcurrants then the cysteine conjugate of 4-methoxy-2-methy-2-butanethiol (10) would have the chemical structure shown below.



10

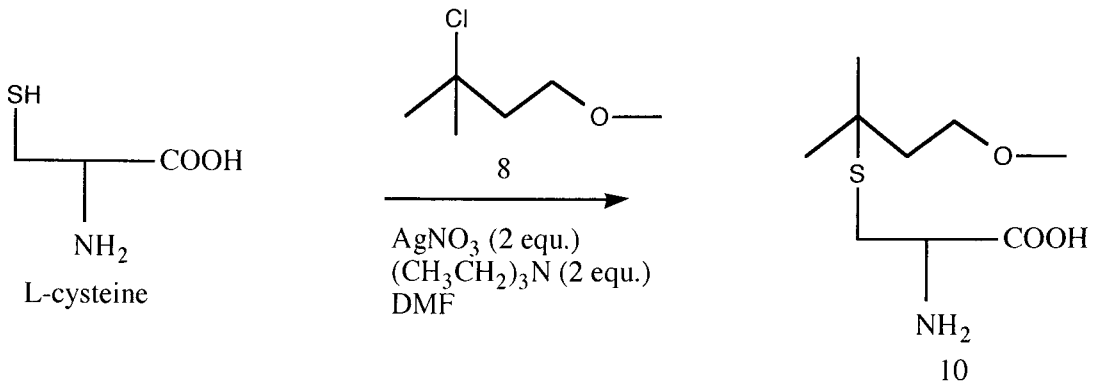
This compound was synthesized using 2-chloro-2-methyl-4-methoxybutane (section 2.3.1, stage 1) and in later experiments the cysteine conjugate was also synthesised using 4-methoxy-2-methyl-2-butanol (section 2.3.2, stage 1)

2.4.1. Synthesis of the Cysteine Conjugate of 4-Methoxy-2-methyl-2-butanethiol

2.4.1.i. Synthesis using 2-Chloro-2-methyl-4-methoxy-butane.

The starting product, 2-chloro-2-methoxy-2-methylbutane, had been previously synthesized (section 2.3.1). Several methods for the synthesis of the cysteine conjugate using this chemical as the starting product were trialed.

Method 1.

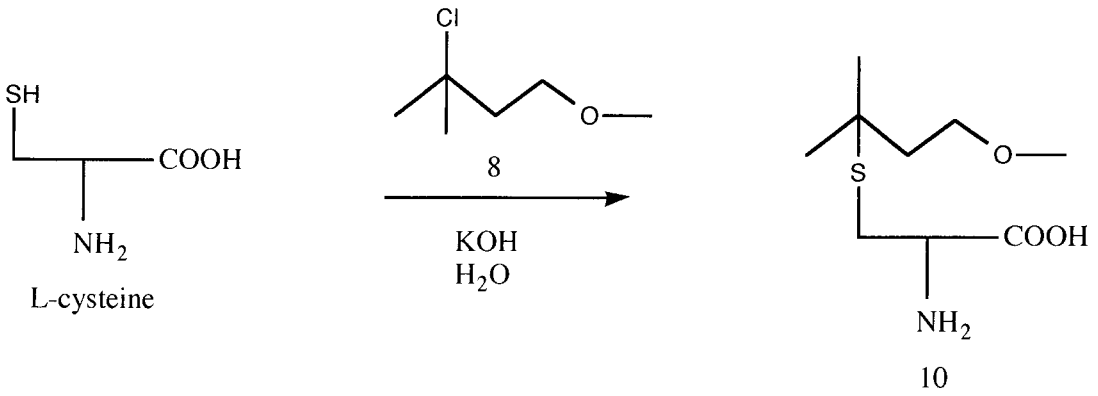


Scheme 5

The first method trialed exploited the reactivity of the tertiary halogen atom in 2-chloro-2-methoxy-2-methylbutane. The formation of a reactive carbocation is promoted by the addition of silver nitrate solution which exploits the affinity of the halide to the solvated silver ion. The transitional carbocation may then react with the thiol group of L-cysteine to form the conjugate.

L-Cysteine (Sigma Aldrich) (75.4 mg, 0.6 mmoles) was mixed with 159.8 mg of silver nitrate (BDH, AnalaR, Aus.) (2 equivalents) and 168 μL of triethylamine (BDH, Port Fairy, Aus.) 2 equivalents of the base were used to deprotonate the carboxylic acid on the cysteine) in 1 mL of dimethylformamide, an aprotic solvent. The 2-chloro-4-methoxy-2-methylbutane was added (100 μL) and the solution was left for 2 hours.

Method 2.

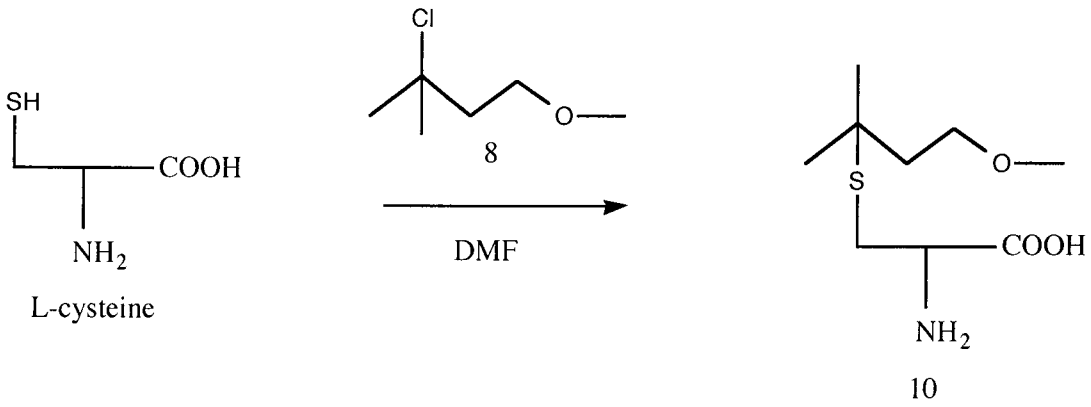


Scheme 6.

The basic potassium hydroxide solution promotes a nucleophilic addition of the nucleophilic sulphur atom in the L-cysteine ion to the tertiary cabocation of 4-methoxy-2-methylbutane.

L-cysteine (Sigma Aldrich) (73.3 mg) was mixed with 0.1 g of potassium hydroxide in 1 mL of water. The 2-chloro-4-methoxy2-methylbutane was added (100 μL) and the solution was left for 2 hours.

Method 3.



Scheme 7

The use of the aprotic solvent, dimethyl formamide may serve to further promote the nucleophilic addition of L-cysteine to the carbocation of 2-chloro-4-methoxy-2-methylbutane by ensuring the sulphur anion of L-cysteine remains unencumbered and therefore more reactive.

L-Cysteine (72.1 mg) was mixed with 1 mL of dimethylformamide (UniChrom, Ajax Finechem, Aus.) and 100 μL of 2-chloro-2-methyl-4-methoxybutane was added. The mixture was left for 2 hours at room temperature.

Method 4.

The increased solvation of 2-chloro-4-methoxy-2-methylbutane in an aqueous alcohol solution dissolves anions and carbocations strongly, facilitating contact between the moderately reactive species.

L-Cysteine (95.1 mg) was mixed with 0.5 mL of 50 % aqueous ethanol and 100 μL of 2-chloro-2-methyl-4-methoxy butane was added. The mixture was agitated then left for 2 hours at room temperature.

Analysis by liquid chromatography mass spectrometry

Separation of components was achieved using Waters 2690 high pressure liquid chromatograph (HPLC) equipped with a Water Nova-Pak C_{18} column of dimensions 390 and 150 mm. The mobile phase was established at 0.6 $mLmin^{-1}$ of 60 % of 1 % acetic acid in 40 % methanol and increasing to 80 % methanol over 15 minutes. The HPLC was coupled to a Finnigan LCQ ion trap mass spectrometer and analyte ionization was achieved with an electrospray ion source (ESI)

ESI conditions:

Source Voltage (kV):	6
Sheath Gas Flow Rate (psi):	100
Aux Gas Flow Rate (psi):	34.00
Capillary Voltage (V):	1.5
Capillary Temp (°C):	260

Analyses using HPLC daughter/daughter ion mass spectrometry (MS/MS) showed method 2 to be the most successful in the synthesis of the 4-methoxy-2-methyl-2-butanethiol cysteine conjugate. The sample pHs were lowered to 7 by drop-wise addition of 0.1 N HCl prior to introduction of the samples into the HPLC MS/MS.

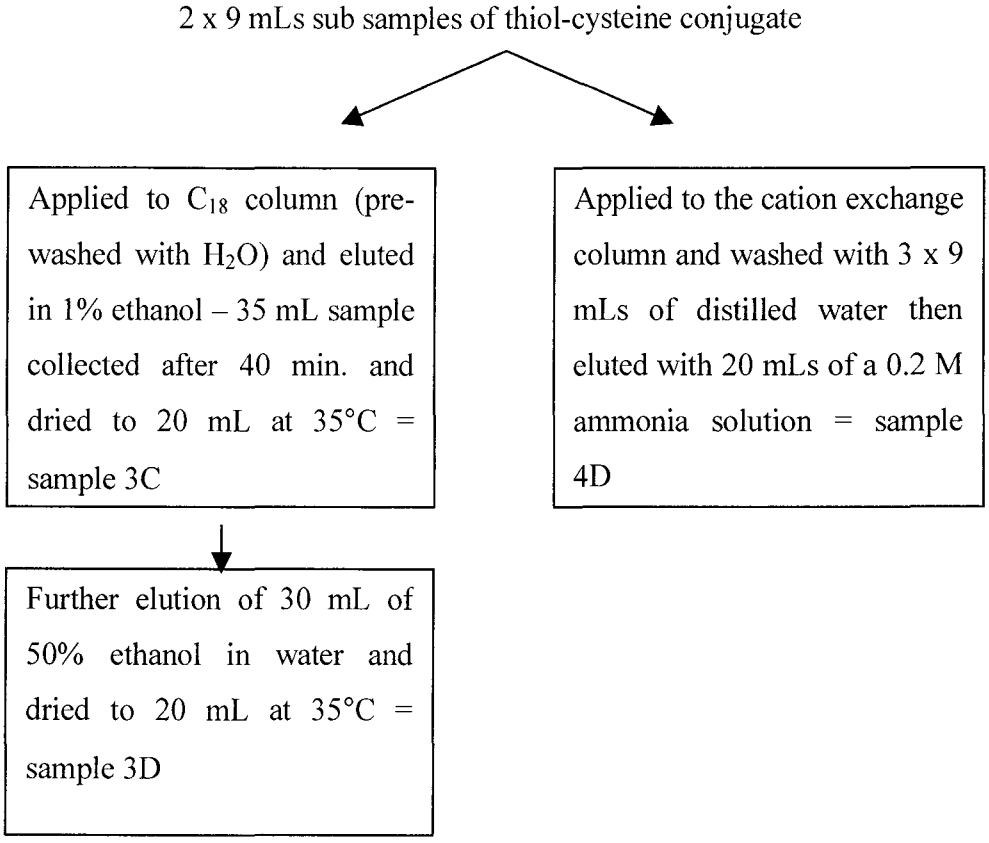
The full MS/MS spectrum from the analysis of the target chemical synthesised using method 2 was obtained from the peak eluting at 3.13 minutes and was generated from the fragmentation of ion m/z 222 using a collision energy of 18 % and an isolation window of 5 m/z units.

2.4.1 ii. Clean-up of the Synthetic 4-Methoxy-2-methyl-2-butanethiol Cysteine Conjugate Synthesized from the 4-Methoxy 2-chloro 2-methyl butane

Several methods to purify the conjugate were trialed.

1. L-cysteine (72 mg) was mixed with 0.1 g of potassium hydroxide in 1 mL of water. The chlorinated methyl methoxy butane was added (100 μL) and the solution was left for 2 hours. The solution was taken to a pH of 7.14 with 1 mL of 1.8 M HCl and the mixture was filtered to remove the white precipitate. The filtrate was applied to a 5 cm C_{18} silica gel (Aldrich) column prepared in 1 % ethanol in water. The column was eluted with 1 % ethanol at a flow rate of 0.5 $mLmin^{-1}$. The target conjugate was not detected in any of the samples subsequently collected over a period of 1.5 hours, including the initial void volume wash. Methanol (Chrom AR, HPLC, Mallinckrodt) (100 %) was passed through the column and collected. The conjugate was detected at low levels in the eluted methanol.

2. L-cysteine (72 mg) was mixed with 0.1 g of potassium hydroxide in 1 mL of water. The chlorinated methyl methoxy butane was added (100 μL) and the solution was left for 2 hours. The solution was taken to a pH of 4 with 17.5 mL of 1N HCl. The aqueous solution was washed with 5 x 15 mLs of dichloromethane (Chrom AR, HPLC, Mallinckrodt). A sub-sample of 0.5 mL was taken and the remaining solution was divided into 2 experiments. A flash chromatographic column was prepared as follows. Dowex 50W-X8 cation exchange resin (20-50 US mesh (H) Aldrich) was washed with 2 M HCl and rinsed with water. It was then washed with 2M NaOH on a magnetic stirrer for 20 minutes and again rinsed with water until the wash solution was neutral and salt free. The resin was then soaked overnight in a large beaker and then the slurry was poured into a plastic column (1 x 1.5 cm) with a scintered glass base.



The largest peak was detected in sample 3C however the relative proportions of daughter peaks in the mass spectrum indicated that some changes in the conjugate structure may have occurred and the clean-up method was considered un-workable.

2.4.1.iii. Synthesis of the Cysteine-thiol Conjugate Using 4-Methoxy 2-methyl butan-2-ol as the Starting Product.

The protic solvent, trifluoro acetic acid (CF_3COOH), promotes the formation of a carbocation directly from the alcohol by protonation followed by the loss of H_2O. The alcohol group on the 4-methoxy-2-methyl-2-butanol is open to such attack allowing the nucleophilic thiol group on the cysteine to bond to the tertiary carbon cation.

4-Methoxy-2-methyl-2-butanol (1 mL) was dissolved in 15 mL of trifluoro acetic acid (Anal. Reag. Ajax Finechem., Aus.). L-cysteine (0.97 g) was added and the solution was kept at 20-30°C overnight. The trifluoro acetic acid was removed by RVE and 90 mL of diethyl ether was added. The solution was taken to a pH of 5 with a 10 % solution of sodium carbonate ($NaCH_3COO^-$).

2.4.2. Development of an Extraction and LC MS/MS Method for the Detection of the Cysteine-thiol Conjugate.

2.4.2.i. Flash Chromatography for the Isolation of Endogenous Cysteine-thiol Precursor from Blackcurrant Buds with 1% Ethanol in Water as the Mobile Phase.

A chromatographic column (2 cm diameter x 7 cm) was prepared by mixing C_{18} silica gel (Aldrich) in ethanol. The resulting slurry was introduced through a funnel onto the scintered glass base of the column. The slurry was left to settle overnight then the column was rinsed with 60 mLs of distilled water and 55 mLs of 1 % ethanol in distilled water.

Frozen blackcurrant buds (100 g) from the HTC variety were ultra-turrexed in 400 mLs of ethanol and filtered through a Buchner funnel equipped with a #4 filter paper. The filtrate (350 mLs) was reduced by 50 % using RVE at 45°C and 140 mLs of water was added. This was transferred to a separating funnel and washed with 3 x 200 mLs of hexane. The cloudy/brown ethanol layer was dried down on the RVE at 45°C. The cloudy suspension changed to a clear pink solution above a brown suspension and 3 x 5 mL of water was added. The mix was filtered through a Buchner (#4 filter paper) to remove the brown suspension and the filtrate was applied to the C_{18} column. The sample was eluted with 1 % ethanol in distilled water by establishing a flow rate of 0.5 $mLmin^{-1}$ and the sample was collected after 45 minutes (column void volume ~25 mL). A 60 mL fraction was collected and evaporated to dryness by RVE at 72°C.

2.4.2.ii. The Inclusion of Antioxidants in the Extraction of Thiol-conjugate from Blackcurrant Buds.

The cysteine-thiol conjugate was not detected in blackcurrant extracts using the procedures described in section 2.4.2.i. Within the wine industry, grapes are preserved with antioxidants and acidification agents to ensure the stability of the juice. In addition, maintaining a pH of 3 is considered critical for stability through the processing stages. Sodium metabisulphite, ascorbic acid and tartaric acid were applied in the extraction process of blackcurrant buds to preserve the cysteine thiol-conjugate.

Preparation of the additive solution - An additive solution was prepared by dissolving 20.2 mg of sodium metabisulphite (Univar), 20.8 mg of ascorbic acid(Sigma Aldrich) and 403.5 mg of tartaric acid (Sigma Aldrich) in 200 mLs of distilled water. The pH was to 2.5.

Synthesis of the cysteine-thiol conjugate - The cysteine-thiol conjugate was prepared by dissolving 0.1 g of potassium hydroxide in 1 mL of water with 79.3 mg of cysteine. A 100 µL aliquot of 4-methoxy-2-methyl-2-butanol was added and the solution was agitated. To improve the miscibility between the non-polar 4-methoxy-2-methyl-2-butanol and the aqueous layer, 1 mL of ethanol was added. The solution became homogenous and slightly yellow in color. The solution was agitated then left at room temperature for 2 days. The solution was transferred to a round bottom flask and the pH adjusted to 2 with the drop-wise addition of 2 M HCl. The solution was dried down at 35°C and weighed to produce 0.99 g of solids. The solids were suspended in redistilled ethanol. A large amount of white crystalline material was evident and this was removed by filtration. The filtrate was transferred to a pre-weighed round bottom flask and dried down by RVE at 35°C. The product (0.088 g) was sampled and analysed by HPLC MS/MS and was determined to contain approximately 13 % of the 4-methoxy-2-methyl-2-butanethiol-cysteine conjugate.

Column preparation. Three C_{18} column were prepared by introducing an ethanol slurry of octadecyl functionised silica gel into a plastic column (dimensions 1 x 5 cm). The media was washed with 1 mL of ethanol (flow rate 3 $mLsmin^{-1}$) and 1 mL of 1 % ethanol in distilled water (flow rate 0.53 $mLsmin^{-1}$).

Recovery of the synthetic cysteine-thiol conjugate from blackcurrant buds. – Synthesised cysteine conjugate (75 mg) was dissolved in 10 mL of distilled water and this solution was used to fortify all the samples detailed in this section. The pH was determined to be 1.9. A sub-sample (10 µL) of the conjugate was dissolved in 500 µL of 1 % ethanol in distilled water as a reference (sample A).

A 10 µL sub-sample of the cysteine conjugate standard solution was used to fortify 9 mLs of distilled water. Additive solution (1 mL) was added such that the pH was 3.06.

The solution was applied to one of the C_{18} columns and eluted with 1 % ethanol in distilled water. The eluant was evaporated down to a 500 µL volume with the RVE at a temperature of 35°C (sample B).

Freshly cut blackcurrant buds cv HTC (70.41 g) were fortified with 10 µL of the synthetic conjugate standard solution in a 500 mL Schott bottle that had been flushed with nitrogen. Additive solution (100 mL) was added and the buds were ultra-turrexed for 5 minutes. The Schott bottle was kept in ice throughout this process. Distilled water (50 mLs) was added and the volume was increased to 250 mL with additive solution to maintain the pH at 3. The mix was filtered through a Buchner funnel with #1 filter paper. The pale yellow filtrate was transferred to a stoppered bottle flushed with nitrogen. The pH was adjusted to 3.0 with 1N HCl. The yellow blackcurrant filtrate was placed on the column and a flow rate of 0.5 $mLmin^{-1}$ was established using a peristaltic pump on the outlet. As the filtrate entered the column a pink color was evident. Aqueous ethanol (50 mL of 1 % ethanol in water) was used to elute the sample. The sample was evaporated to reduce the volume to 500 µL by RVE at 40°C (sample C).

Freshly cut blackcurrant buds cv HTC (70.66 g) were weighed into a 500 mL Schott bottle that had been flushed with nitrogen. Additive solution (150 mL) was added and the buds were ultra-turrexed for 5 minutes. The Schott bottle was kept in ice throughout this process. Distilled water (50 mLs) was added and the volume was increased to 250 mL with additive solution to maintain the pH at 3. The mix was filtered through a Buchner funnel with #1 filter paper. The pale yellow filtrate was transferred to a stoppered bottle flushed with nitrogen. The pH was adjusted to 3.0 with 1N HCl. In this extraction the blackcurrant filtrate was a clear pink. The solution was placed on the column and a flow rate of 0.5 mL min^{-1} was established using a peristaltic pump on the outlet. Aqueous ethanol (50 mL of 1 % ethanol in water) was used to elute the sample. The sample was evaporated to reduce the volume to 500 µL by RVE at 40°C (sample D). Results are presented in section 3.4.2.ii..

Re-extraction of endogenous cysteine-thiol conjugate - Further experimentation was undertaken to confirm the identification. New columns were prepared and the

synthetic cysteine conjugate was stored away from sample preparation areas until the extractions of non-fortified samples were completed. In addition the concentrations of the components in the additive solution were increased to try to reduce the possible oxidation of the conjugate observed in sample C. Sodium metabisulphite (76.5 mg), 52.3 mg of ascorbic acid and 1.005 g of tartaric acid were dissolved in 500 mLs of distilled water. The resultant pH was 2.5.

*Sample preparat*ion - Blackcurrant buds cv HTC were harvested on the same day of analyses and were cut from the canes by hand. The samples were stored in plastic bags flushed with nitrogen at 8°C. Buds (70.58 g) were weighed into a 500 mL Schott bottle that had been previously flushed with nitrogen. The bottle was kept semi-submerged in crushed ice. Refrigerated additive solution (150 mL) was added and the buds were ultra-turrexed for 5 minutes. The sample was filtered through a Buchner funnel fitted with a #1 filter paper. The pale pink filtrate was kept under nitrogen. The pH of 3.8 was adjusted to 3.0 with 20 to 30 drops of 1N HCl. The liquid was introduced to the C_{18} column that had been setup in an 8°C cooler room. A peristaltic pump was used to establish a flow of 1 $mLmin^{-1}$. The sample was eluted with 50 mLs of 1 % ethanol in distilled water. The eluant was evaporated to dryness on the RVE at 40°C then taken up in 500 µL of distilled water. The column was washed with 20 mLs of methanol and the wash was also analysed.

A second sample of blackcurrant buds was prepared as described. However 10 µL of the standard solution of the synthetic cysteine conjugate was used to fortify the buds prior to ultra-turrexing. All samples were analysed in conjunction with a reference sample of 10 µL of the synthetic cysteine conjugate in 500 µL of distilled water.

2.4.2.iii. Repeatability of the extraction and detection of the thiol conjugate in blackcurrant buds.

In order to determine if the extraction method developed for the thiol conjugate from blackcurrant buds was quantitative and repeatable the extraction process was conducted in triplicate.

Additive solution. Sodium metabisulphite (154.9 mg), 103.3 mg of ascorbic acid and 2.001 g of tartaric acid were dissolved in 1 L of distilled water.

Column preparation. Three C_{18} columns were prepared by introducing an ethanol slurry of approximately 3.7 g of octadecyl functionalised silica gel into a plastic column (dimensions 1 x 5 cm) that was left to settle overnight. The media was washed with 20 mLs of water, 10 mLs of 1 % ethanol in water, 10 mLs of methanol and 10 mLs of water.

Preparation of blackcurrant buds. Freshly harvested blackcurrant buds cv HTC were cut by hand from blackcurrant canes. Four samples (10 g) of buds were weighed and dried at 75°C and re-weighed to determine the dry weight of buds. The remaining fresh buds were stored under nitrogen overnight. As the responses recorded for previous extracts were well above the detection limit of the HPLC MS/MS, sample sizes were reduced to 25 g. Three samples of blackcurrant buds (~25 g) were placed in 250 mL Schott bottles that had been flushed with nitrogen and placed on crushed ice. Additive solution (50 mLs) was added and the samples were ultra-turrexed for 5 minutes and filtered through Buchner funnels each fitted with #1 filter paper. The filtrates were transferred to clean, nitrogen filled 250 mL Schott bottles and the pH was adjusted to 3.0 with 1N HCl added drop-wise (15-25 drops). The bottles were flushed with nitrogen and stored in a cool room at 4°C. The colour of the extracts varied from clear pink, cloudy pink and yellow pink. The three samples were loaded onto C_{18} columns and eluted with 30 mLs of 1 % ethanol. The eluants were evaporated to dryness at 40°C on the RVE and made up in 500 µL of distilled water. A standard of 10 µL of the cysteine-thiol conjugate previously prepared (section 2.4.2.ii) was dissolved in 500 µL of distilled water.

Analysis of the endogenous thiol. The buds collected for the repeatability experiment were also processed to produce a solvated extract (section 2.1.1.iv) and analysed by GC FPD (section 2.1.1.iii).

2.4.2.iv. The Repeatability of the Extraction of the Cysteine-thiol Conjugate When Flash Chromatography is Excluded.

Blackcurrant canes cv HTC from the University of Tasmania farm were harvested and the buds cut by hand. The buds from the clones were combined and well mixed. Sub-samples were taken for dry bud weight calculations. A further 3 x 40 g sub samples were also taken to be analysed by GC FPD (section 2.1.1). Three ~30 g samples were weighed into 250 mL Schott bottles that had been previously cooled and flushed with nitrogen. Aliquots of cooled additive solution (65 mLs) were added and the solutions were ultra-turrexed for five minutes under a nitrogen atmosphere. The samples were filtered through Buchner funnels each fitted with a #1 filter paper and the filtrates were returned to clean, nitrogen filled, cold Schott bottles. The pHs were adjusted to 3.0 with 0.1N HCl. The samples were then taken to dryness on the RVE at 40°C and made up in 500 μL of distilled water. The samples were thick and an extra 500 μL of water was added to each. Sub-samples (500 μL) were transferred to plastic Erlenmeyer tubes and centrifuged at 2000 rpm for 5 minutes. A 10 μL synthetic cysteine-thiol conjugate standard (section 2.4.2.i) was dissolved in 500 μL of distilled water and analysed alongside the three replicates by LC MS/MS.

2.4.3. The Relative Concentration of the Cysteine-thiol Conjugate in White Bud and High Thiol Variety Blackcurrant Buds.

The levels of endogenous thiols have been found to be significantly higher in the HTC plants propagated at the University of Tasmania relative to the variety used in most commercial plantation, White Bud. An experiment to determine whether the levels of the cysteine-thiol precursor were also disparate in the two selections was undertaken.

Additive solution – Sodium methabisulphite (0.51 g), 2.06 g of tartaric acid and 0.10 g of ascorbic acid were dissolved in 500 mL of distilled water. The pH was 2.5.

In section 2.2.1. the variations in thiol levels between clones selected for the high thiol content and the corresponding levels in the commercial White Bud variety was detailed. HTC plants from the same trial site that had been established at the University of Tasmania were harvested, the buds cut from the cane by hand and the buds combined. The buds were gently and thoroughly mixed and quadruplicates were

sub-sampled. In addition canes from the remaining White Bud variety grown at the same trial site were harvested, the buds cut from the cane by hand and sub-sampled in quadruplicate. The HTC buds and those from the standard White Bud variety were extracted concurrently and analysed by GC FPD and HPLC MS/MS to determine the relative concentrations of thiol and cysteine-thiol conjugate.

Blackcurrant bud extraction – Each of 30 g samples of blackcurrant buds were weighed into 250 mL Schott bottles which had been previously cooled and flushed with nitrogen. Additive solution (65 mL) was added and the samples were ultra-turrexed for 1 minute. The samples were filtered through Buchner funnels, each fitted with #1 filter paper. Each of the filtrates were quickly transferred to a second cooled Schott bottle that had been flushed with nitrogen and the pH was adjusted to 3 by the drop-wise addition of 1N HCl. The solutions were then transferred to 100 mL round bottom flasks and dried down at 40°C using the RVE. The residues were very dark pink. The samples were taken up in 1 mL of additive solution, transferred to Eppendorf tubes, flushed with nitrogen and centrifuged for 5 minutes at ~ 20 000 rpm. Sub-samples were transferred into 250 µL HPLC vial inserts and submitted for analyses. A 10 µL aliquot of the synthetic standard conjugate (section 2.4.2) was diluted into 500 µL of distilled water and analysed alongside the 2 sets of quadruplicates. Samples were analysed by HPLC MS/MS as detailed below.

Sub-samples of each quadruplicate were also taken and analysed to determine the level of endogenous thiol by GC FPD as detailed in the methods in section 2.1.1.

Analysis by HPLC MS/MS

Separation of components was achieved using Waters 2690 HPLC equipped with a Water Nova-Pak C_{18} column of dimensions 390 and 150 mm. The mobile phase was established at 0.6 $mLmin^{-1}$ of 85 % of 2 % acetic acid in 15 % methanol increasing to 80 % methanol over 20 minutes. The HPLC was coupled to a Finnigan LCQ ion trap mass spectrometer and analyte ionization achieved with ESI.

ESI conditions:

Source Voltage (kV):	5.8
Sheath Gas Flow Rate (psi):	95
Aux Gas Flow Rate (psi):	28
Capillary Voltage (V):	10
Capillary Temp (°C):	240

Divert valve:

Divert flow until (min):	5.20
Resume divert at (min):	7.20

Scan events in each scan cycle;

1: selected reaction monitoring of 101 and 122 from 222, isolation width 5, collision energy 18 %

2: full scan 90 to 500

3: data dependent MS/MS from the most intense ion from previous full scan, 18 % CE.

The results for the GC FPD and HPLC MS/MS analyses of high thiol clones and the White Bud variety are listed in table 3.4.v.

2.4.4. The Monitoring of the Endogenous Thiol and Cysteine-thiol Conjugate in Blackcurrant Buds Prior to Bud Burst.

The final harvest season of experimentation in this study was drawing to a close and despite the need for further method development and validation it was decided to include a time series trial to monitor the levels of the endogenous thiol and the corresponding responses of the cysteine-4-methoxy-2-methyl-2-butanethiol conjugate in blackcurrant buds. The remaining HTC canes established at the trial site at the University of Tasmania farm at Cambridge, Southern Tasmania, were harvested on a weekly basis, up until bud burst.

Sample collection – A similar number of canes were cut from the same sections of the trial plot each week. Buds were cut from the cane by hand and placed in plastic bags

and flushed with nitrogen. The buds were placed in an 8°C cool room until processing. The buds from each section were gently and thoroughly mixed and sub-samples were taken for measurements of bud dry weight. Additive solutions were prepared on each day of analyses.

Additive solution -- Sodium metabisulphite (1.5 g), 2 g of tartaric acid and 0.1 g of ascorbic acid were dissolved on 0.5 L of distilled water.

Quadruplicates of 30 g sub-samples of buds were extracted and analysed as described in section 3.4.3. In addition sub-samples of 10 g were extracted and analysed to determine the level of 4-methoxy-2-methyl2-butanethiol by GC FPD as described in section 2.1.1.

Samples were collected on the 15th, 22nd and 29th of July, on the 5th, 12th, 19th and 26th of August, the 2nd and 21st of September and the 1st and 8th of October.

RESULTS

Section 3.1. HARVEST AND EXTRACTION TECHNOLOGY

3.1.1. Sieving Experiment.

A 14 kg sample of buds harvested from a commercial White Bud plantation was passed through the sieving machine shown in plate 1.

Plate 1. Machine used to sieve blackcurrant buds from chaff.

Plate 2. Examples of fractions collected from sieving machine.

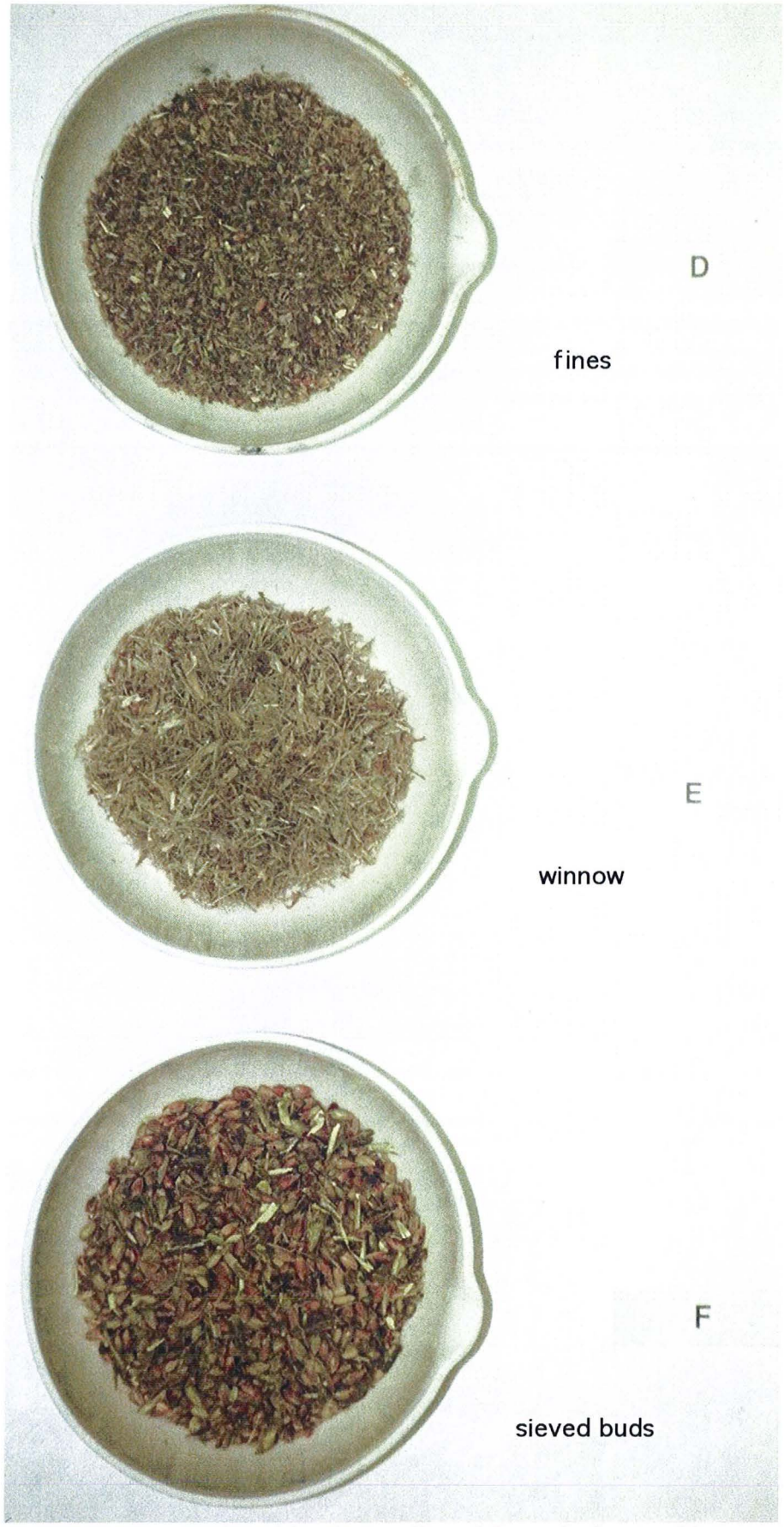


Plate 3. Examples of fractions collected from sieving machine.

The total weight of all fractions collected was 12.9 kg (92 % recovery). Plates 2 and 3 show the consistency of the samples collected. Table 3.1.i. records the weights of the fractions obtained and the oil yields subsequently extracted. Although no replication was undertaken the results are useful as an overview of how extraneous co-harvested material contributes to the extract quality and extraction costs. The results for un-sieved buds are calculated from a smaller sample but extrapolated to reflect the yield that would have been obtained from a 14 kg sample.

sample	un-sieved buds (2)	sieved buds (1)	winnow fraction (3)	#10 sieve (4)	#7 sieve (5)	fines (6)	sum total
	A	F	E	B	C	D	
sample weight (g)	1400	8425	122	342	547	3440	12876
% of combined sample	100	65.4	0.9	2.7	4.2	26.7	100
%dry weight	41.6	43.7	73.3	51.5	49.8	45.1	
oil yield (g)*	277	246	1	0.5	0.7	42.7	290.8
% oil yield	1.98	2.92	0.82	0.14	0.12	1.24	2.26
thiol $mgkg^{-1}$ in oil	8.6	9.9	2.7	0.8	0	3.4	

*extrapolated from 50 g sub-sample extraction

Table 3.1.i. Weight & Oil Yield from Buds Processed Through a Sieving Machine.

The results indicate that machine harvested samples are partly composed of large pieces of cane shards, sticks and extraneous material (fractions E+B+C) that accounts for 8 % of the total weight and therefore increases the solvent required by 8 % whilst yielding only 0.7 % of the oil. In addition it was observed that the winnow (fraction E) completely absorbed the solvent that had been added at a ration of 4 x w/v. Very little solvent was recovered. Removal of this highly absorbent material may reduce further the amount of solvent required for effective extraction of sieved buds.
The yield of individual components is calculated by relating the area of the relevant peak detected by GC FID to the area of a known weight of internal standard (assuming a 1 : 1 response ratio) using the formula;

$$\text{mg component} = \frac{\text{area of component}}{\text{area of } C_{18}H_{38} \text{ internal standard}} \times \text{mg of } C_{18}H_{38} \text{ internal standard added}$$

The amount detected in the sub-sample (20-50 mg) is then extrapolated to reflect the concentration in the original sample by applying the formula;

$$\text{mg of component in total sample} = \text{mg in sub-sample} \times \frac{\text{weight of total sample (mg)}}{\text{weight of sub-sample (mg)}}$$

The yield of components detected by GC FID for each sample type is listed in table 3.1.ii. The identification of the components was based on retention times and elution patterns previously established (Kerslake, 1984; Poulter, 1991).

component	mg component/kg of bud DMB					
	un-sieved buds (2)	sieved buds (1)	winnow fraction(3)	#10 sieve (4)	#7 sieve (5)	fines (6)
		F	E	B	C	D
%recovered	100	65	1	3	4	3
α-thujene	19	27	8.5	0.0	0.0	15.1
α-pinene	14	31	1.4	2.0	0.6	6.0
sabinene	383	744	52.3	3.9	1.6	188.5
mycrene	10	19	1.3	0.0	0.0	4.5
δ-3-carene	236	424	27.0	0.0	0.7	97.8
β-phelllandrene +limonene	131	175	11.9	0.0	0.6	43.2
ocimene	20	47	0.9	0.0	0.3	6.4
terpinolene	135	219	5.9	1.8	0.3	48.8
β-caryophyllene	172	269	17.8	7.8	2.9	84.9
humulene	55	88	4.7	2.3	0.9	27.6
germacrene-D	65	107	1.1	0.0	0.0	25.4
bicyclogermacrene	11	17	0.0	0.0	0.0	3.5
caryophyllene oxide	26	42	13.4	10.9	3.7	44.3
hardwickic acid	291	452	0.0	0.0	0.0	120.6
20-oxo-hardwickic acid	75	115	0.0	0.0	0.0	36.6

Table 3.1.ii. Amount of Each Component ($mgkg^{-1}$ DMB) in Sieved and Un-sieved Buds.

Table 3.1.ii. shows that the volatile components from processed buds are most concentrated in the sieved buds (F) and in the fines (D). The highest concentration of endogenous thiol was in the sieved buds that are also higher in almost all of the GC amenable chemicals. Concentrations of quality components such as β-caryophyllene and other sesquiterpenes are around 1.5 fold the concentration detected in un-sieved buds. Many monoterpenes in the sieved buds are almost double the concentrations detected in the un-sieved.

Despite the higher levels of many components extracted from sieved buds the relative compositions of the extracts from sieved and un-sieved buds are similar. The proportion of each component within the final extract is calculated using the formula;

$$\% \text{ component in extract} = \frac{\text{weight component calculated (mg)}}{\text{weight of oil analysed (mg)}} \times 100$$

The %composition of the volatiles in the extracts are presented in table 3.1.iii.

component	% component in blackcurrant extract					
	un-sieved buds (2)	sieved buds (1)	winnow fraction(3)	#10 seive (4)	#7 sieve (5)	fines (6)
α-thujene	0.04	0.04	0.07	0.00	0.00	0.05
α-pinene	0.03	0.04	0.01	0.07	0.03	0.02
sabinene	0.83	1.05	0.45	0.14	0.07	0.67
mycrene	0.02	0.03	0.01	0.00	0.00	0.02
δ-3-carene	0.51	0.60	0.23	0.00	0.03	0.35
β-phellandrene +limonene	0.28	0.25	0.10	0.00	0.02	0.15
ocimene	0.04	0.07	0.01	0.00	0.01	0.02
terpinolene	0.29	0.31	0.05	0.06	0.01	0.17
β-caryophyllene	0.37	0.38	0.15	0.28	0.12	0.30
α-humulene	0.12	0.12	0.04	0.08	0.04	0.10
germacrene-D	0.14	0.15	0.01	0.00	0.00	0.09
bicyclogermacrene	0.02	0.02	0.00	0.00	0.00	0.01
caryophyllene oxide	0.06	0.06	0.12	0.39	0.15	0.16
hardwickic acid	0.63	0.64	0.00	0.00	0.00	0.43
20-oxo-hardwickic acid	0.16	0.16	0.00	0.00	0.00	0.13

Table 3.1.iii. The Percentage of Each Component Detected in the Extract of Sieved and Un-sieved Buds.

Although the results are qualitative the indications are that that the quality of the extract obtained from sieved buds would not be compromised.

3.1.2. Solvent and Maceration Methods

Detailed in the section 2.1.1 in Material and Methods are the general analytical methods used in a majority of experiments undertaken in this section. Assessments as to whether alterations to extraction protocols are an improvement on the normal procedures are based on the %yield, the color, consistency and aroma of the extract and the quantification of components by GC. Obviously to facilitate a complete assessment blackcurrant extract must be produced by removal of extracting solvents. However it was anticipated that in larges scale harvest and incubation experiments outlined in section 2.2, production of the final blackcurrant product was not feasible. Where concretes are not produced and the solvated extract is analysed, the total yield is calculated by relating the sum of the areas of the peaks detected by GC FID to the area of a known weight of internal standard (assuming a 1 : 1 response ratio) using the formula;-

$$\text{mg of concrete detected by GC FID} = \text{total area of peaks} \times \frac{\text{mg internal standard}}{\text{area of internal standard}}$$

In order to compare the yields of solvated extracts calculated using the above formula and the yields obtained from weight measurement of extracts from which solvent had been removed by RVE, experiments were established and are detailed in section 2.1.3.

In addition to defining the relationship between different sub-sampling techniques, a series of simple extraction experiments were undertaken to evaluate the effect on extract composition of grinding buds in air and under nitrogen. In addition the effect of solvent composition on extract quality and yield were also studied.

Table 3.1.iv records the yields determined for each extraction method. In addition the yields of volatiles produced by each extraction method are listed. The volatiles are defined as all components eluting prior to the peak recorded for the internal standard

octadecane. No thiols were detected in the extracts of the blackcurrant buds. This was most likely due to the loss of thiol during the storage of the buds which were sourced from the previous harvest season.

method of maceration (n=5)		solvent	%yield of concrete±SD (based on weight)	%yield of concrete.±SD (calculated from FID)	%volatile relative to buds ± SD (calculated)	%yield of concrete relative to method 1
rolling	1	5%hex.in pent.	3.1±0.3	1.15±0.09	0.45±0.07	100
ginding liq.N_2	2	100% hexane		0.51±0.06	0.19±0.02	44
ginding liq.N_2	3	5%hex.in pent.		0.6±0.1	0.26±0.06	52
ginding liq.N_2	4	1%hex.in pent.		0.7±0.1	0.30±0.05	61
stomacher	5	1%hex.in pent.		1.7±0.2	0.68±0.05	148

Table 3.1.iv. Recoveries of Components from Blackcurrant Buds Using Different Methods of Maceration and Varying Solvent Combinations.

The percentage yield of concrete produced from blackcurrant buds using the standard extraction procedure was 3.1 % ± 0.3, much higher than the 1.15 % ± 0.09 which is detected in the solvent of the same sample and calculated using the formula described above. It is evident that GC FID has only detected 37 % of the components of blackcurrant extract produced using the commercial method. The remaining 63 % must be non-volatile components or components not amenable to analysis by GC. Any inferred conclusions drawn from the results obtained from samples that have not been subject to the complete extraction process must take into consideration the disparity between actual and calculated results.

Extracting in 100 % hexane with no dry down gave the lowest recovery with only 44 % recovery relative to the recovery calculated for buds which had undergone the full standard extraction protocol. The best recoveries were achieved using the stomacher (148 % recovery), with the concomitant higher yield of volatiles, an indication the quality was not compromised using this maceration technique. However, the stomacher presented logistical problems. The solvents dissolved the plastic bags used and the technique is one not easily adapted to industrial processes.

3.1.3. Effect of Homogenisation Under Solvent Using an Ultra-turrex

The impracticalities of grinding of blackcurrant buds under liquid nitrogen in a mortar and pestle (methods 2 to 4, section 3.1.2) warranted the assessment of alternative methods of homogenisation. The recoveries of components from blackcurrant buds using the mortar and pestle (method A) were compared to that from the homogenisation of buds under solvent using an ultra-turrex (method B). Table 3.1.v compares the recoveries for the two extraction methods.

method (n=3)	calculated %yield (±SD) of concrete	%yield (±SD) of volatiles relative to buds	$mgkg^{-1}$ thiol (±SD) relative to buds
A	1.65±0.08	0.67±0.07	7.2±0.8
B	3.7±0.6	1.3±0.2	11±1

Table 3.1.v. Recoveries of Blackcurrant Components from Extraction Method A (grinding under liquid nitrogen) and Method B (ultra-turrexing buds in solvent)

Results show that direct homogenisation into the solvent (method B) was the more effective method for extraction of components from blackcurrant buds returning an overall recovery of 3.7 % compared to the 1.65 % recorded from buds that had been ground under liquid nitrogen. The yields of individual components from the two methods are listed in table 3.1.vi.

component	method A mortar & pestle	method B ultra-turrex
(n=3)	%yield x 10^{-3} (±SD)	%yield x 10^{-3} (±SD)
α-thujene	6.3±0.1	12±1
α-pinene	9±1	18±3
sabinene	298±34	565±108
mycrene	19±2	37±7
δ-3-carene	110±13	212±39
β-phellandrene + limonene	22±3	40±7
ocimene	17±2	33±6
terpinolene	53±6	102±18
β-caryophyllene	33±3	56±7
humulene	6.9±0.4	11±2
germacrene-D	0	0
bicyclogermacrene	77±5	126±18
caryophyllene oxide	14.7±0.5	25±2
(-) polyalthic acid methyl ester	132±4	267±48
hardwickic acid methyl ester	663±27	1786±299
20-oxo-hardwickic acid methyl ester	77±5	242±26

Table 3.1.vi. %Yield of Components from Blackcurrant Buds Extracted Using a Mortar and Pestle (method A) and an Ultra-turrex (method B).

The components listed show that in the majority of cases the use of an ultra-turrex to mascerate buds in solvent resulted in a two fold increase in yield. For hardwickic acid and 20-oxo-harkwickic acid (both derivatised to the esters using diazomethane) the yield has increased by three fold. This would indicate that these components are either labile when exposed to air during masceration or ultra-turrexing has allowed for increase sample penetration and improved release of components from cellular structures.

3.1.4. Preliminary Experiments to Include the Steeping of Buds in Ethanol.

Ethanol was introduced into the extraction process with view to improving solvent penetration into blackcurrant buds and retarding oxidative and enzymatic processes. Ethanol extracts however, produce thick dark tarry products that are unacceptable to

the market. However, by partitioning ethanol extracts into non-polar solvents, the essential extraction process currently used by industry is still retained, whilst encorporating the potential advantages of ethanol. To this regard we undertook the steeping of buds in ethanol and partitioned the extracted components into 5 % hexane in petroleum ether. The effect of the inclusion of ethanol in the extraction process was compared to the standard solvent protocols. In preliminary experiments a 2 x v/w ethanol to bud ratio was determined to be sufficient to cover the blackcurrant buds.

Within the experiment reported in the following pages, a third extraction was undertaken to include the solvent, ethyl acetate, in the extraction process. Ethyl acetate had been recommended by European colleagues to be beneficial in the extraction of quality components from blackcurrant buds (pers. comm. R. C. Menary).

For the results presented in this section the amount of any one component (mg) in the oil sub-sampled for analyses were calculated as described in section 3.1.1. This was related to the total amount extracted by multiplying by the weight of total concrete obtained in the extraction and dividing by the amount sub-sampled. This was in turn related back to the weight of buds extracted on a dry matter basis (DMB) using the calculation:-

$$\% \text{ yield of component} = \frac{\text{total mg of component extracted}}{\text{weight of buds extracted (DMB)}} \times 100$$

Table 3.1.vii records the results obtained by GC FID.

Extraction Method	1			2			3		
	5% hex in petroleum ether			ethanol based			ethanol/ethyl acetate		
% yield DMB	rep1	rep2	**mean**	rep1	rep2	**mean**	rep1	rep2	**mean**
oil (by weight)	5.9	6.4	**6.2**	5.4	4.8	**5.1**	4.8	6.0	**5.4**
volatiles	1.2	1.5	**1.4**	1.9	1.7	**1.8**	0.4	0.3	**0.4**
acids	2.3	2.3	**2.3**	1.5	1.3	**1.4**	1.5	1.6	**1.5**
%unaccounted by GC	2.4	2.6	**2.5**	2.0	1.8	**1.9**	2.9	4.1	**3.5**
%component($x10^{-3}$)									
thujene	8.7	11.6	**10.1**	10.2	8.5	**10.2**	4.6	4.8	**4.7**
α-pinene	23	43	**33**	350	306	**328**	1.8	2.6	**2.2**
sabinene	211	317	**264**	227	179	**203**	14.2	8.7	**11.5**
myrcene	30	40	**35**	59	50	**54**	4.7	1.5	**3.1**
δ-3-carene	247	322	**284**	239	195	**217**	14.0	4.7	**9.3**
β-phellandrene & limonene	179	212	**195**	304	267	**285**	52	28	**40**
cis-β-ocimene	30	36	**33**	52	45	**49**	10.6	4.8	**7.7**
terpinolene	130	147	**138**	113	92	**102**	33	17	**25**
β-caryophyllene	145	154	**149**	115	98	**106**	89	88	**88**
α-humellene	39	42	**41**	28	24	**26**	23	23	**23**
germacrene D	48	54	**51**	37	32	**34**	32	32	**32**
bicyclogermacrene	10	12	**11**	12	10	**11**	12	12	**12**
carylophyllene oxide	21	19	**20**	14	12	**13**	10.1	9.4	**9.8**

‡small fraction of part of sample 1 lost

Table 3.1.vii. Yield of Concrete and Components (DMB) Extracted from Blackcurrant Buds.

The results for method 1 are not directly comparable to the other three methods as the buds were not homogenised in a blender but rather rolled frozen and extracted in solvent on a shaker bath. However some basic conclusions may be drawn. The yield of volatiles is higher in the ethanol extraction method 2. Surprisingly there were more acids extracted using the 5% hexane in petroleum ether (method 1) than using ethanol relative to the weight of buds extracted DMB. Yet method 2 included, not only a more penetrating method of homogenisation, but also used solvents which were significantly more polar. In addition 40 % of the extract produced using method 1 was not detected by GC FID compared to 37 % of that produced using method 2. The most marked

difference was in the levels of α-pinene extracted with method 2 producing 10 x the level of that recorded for extraction method 1. It may be predicted that this would impact dramatically on the aroma of the concrete produced. The homogenisation in ethanol also resulted in elevated levels of myrcene, β–phellandrene and limonene (co-eluting) and ocimene

The inclusion of ethylacetate in the extracting solvent (method 3) was not effective. Exceedingly low levels of volatiles were extracted.

3.1.5. Effect of Filtering Extracting Solvent from Buds Prior to Partitioning into Non-polar Solvents

In the previous experiment the buds were removed from the preliminary extracting solvent, ethanol, prior to extaction using the solvent most commonly used in the blackcurrant industry, 5 % hexane in petroleum ether. The extracting, non-polar solvent was only in contact with the buds prior to partitioning, with subsequent washes used only to extract components from the ethanol layer and it was considered that components may have still been present in the buds. Further investigation was undertaken to determine whether the decanting of the polar extracting solvent from the buds, prior to the instigation of the partition step, significantly altered the product obtained.

In the previous experiment, the 2 x v/w of ethanol to bud ratio was only sufficient to cover the buds. In this experiment the solvent volume was increased to 3 x v/w ethanol to bud ratio. Preliminary tests (experiment not reported) showed that with the increase in ethanol volume, the formation of a partition was spontaneous and the addition of water was not required. A final wash of the extracted buds with 5 % hexane in petroleum ether was also undertaken to further improve product yield.

Table 3.1.viii lists the results for the extraction of blackcurrant using the methods outlined. The results for the two stages of the extraction process, namely the extraction of buds in ethanol followed by a non-polar partition extraction and the production of a marc from the extracted buds, were combined.

Filtering the ethanol from the buds prior to the partitioning of components from the polar aqueous layer to the non-polar extracting solvent resulted in higher yields. However, based on the total amount of material detected by GC FID relative to the weight of material actually introduced into the GC, method 2 produced extracts that contained 11 % more of the type of chemicals that were not amenable to the GC than method 1 and these would be less likely to contribute to the aroma profile. Higher levels of the acids, polyanthic, hardwickic and 10-oxo-hardwickic were also apparent in extracts produced using method 2. The un-filtered extracts appeared greener and thicker and aroma assessments identified that these extract had an attractive fruity and fresh aroma. The extracts produced from the process whereby the polar layer was partitioned into the non-polar after the buds had been removed were found to be more catty with strong a blackcurrant herbaceous background. It would appear that the buds should not be in contact with the non-polar extracting solvent through the process of partitioning.

	method 1 not filtered			method 2 filtered		
	rep1	rep2	**mean**	rep1	rep2	**mean**
thiol ($mgkg^{-1}$)	1.49	1.46	**1.47**	2.39	2.44	**2.42**
%Yields relative buds (DMB)						
concrete	6.22	6.38	**6.3**	7.25	6.88	**7.1**
volatiles	0.76	0.72	**0.74**	0.76	0.72	**0.74**
acids	2.27	2.32	**2.3**	1.9	2.2	**2.0**
not amenable to GC	51.3	52.3	**51.8**	63.6	58.0	**60.9**
	%yield $x10^{-3}$(DMB)			%yield $x10^{-3}$(DMB)		
thujene	8	8	**8**	8	8	**8**
α-pinene	3	7	**5**	11	5	**8**
sabinene	88	91	**90**	126	91	**109**
myrcene	14	26	**20**	18	16	**17**
δ-3-carene	119	81	**100**	130	105	**118**
β-phellandrene & limonene	70	90	**80**	100	88	**94**
cis-β-ocimene	20	20	**20**	20	19	**20**
terpinolene	86	94	**90**	78	76	**77**
β-caryophyllene	135	124	**130**	104	123	**113**
humulene	33	40	**37**	28	35	**32**
germacrene D	45	49	**47**	32	34	**33**
bicyclogermacrene	14	13	**13**	7	4	**5**
carylophyllene oxide	15	17	**16**	15	17	**16**
polyanthic acid methyl ester	187	185	**186**	143	167	**155**
hardwickic acid methyl ester	1850	1750	**1800**	1368	1592	**1480**
20-oxo-hardwickic acid methyl ester	174	175	**174**	141	167	**154**

Table 3.1.viii. Component Recoveries from Buds Partitioned Between Polar and Non-polar Solvents With Buds Present (method 1) and Not Present (method 2)

3.1.6. The Inclusion of Propylene Glycol in the Extracting Solvent.

The inclusion of propylene glycol, a solvent of low volatility, may prevent the loss of low volatile components during the extraction of blackcurrant buds. Propylene glycol was included in the extracting solvent. Ratios trialed were 1 : 1 and 0.2 : 1 propylene glycol to bud weight (v:w) in the ethanol extracting solvent. The extracts produced

using the two methods were darkly colored, lacking viscosity but possessing deep fruity aromas. Table 3.1.ix records the yields of the relevant extraction protocols.

%yield of components	standard ethanol method			method 1 1:1 prop. glycol (v:w)			method 2 0.2:1 prop. glycol (v:w)		
	rep1	rep2	mean	rep1	rep2	mean	rep1	rep2	mean
thiol ($mgkg^{-1}$)	0.65	0.64	0.64	0.78	0.97	0.88	1.23	1.41	1.31
concrete	7.3	6.9	7.1	8.0	9.6	8.8	8.8	10.8	9.8
volatiles	0.8	0.7	0.7	0.8	1.2	1.0	0.70	0.66	0.68
acids	1.9	2.2	2.0	1.6	2.0	1.8	1.6	2.2	1.9
not amenable to GC	63.6	58.0	60.9	69.7	66.3	67.8	73.7	73.3	73.5
	%yield $x10^{-3}$(DMB)			%yield $x10^{-3}$(DMB)			%yield $x10^{-3}$(DMB)		
thujene	7.7	8.4	8.0	5.8	8.9	7.4	4.1	4.3	4.2
α-pinene	11.1	5.5	8.3	17.1	28.2	22.6	9.2	7.4	8.3
sabinene	126	91	109	143	233	188	102	88	95
myrcene	17.9	15.6	16.7	20.1	30.0	25.1	14.7	13.5	14.1
δ-3-carene	130	105	118	156	235	195	119	111	115
β-phellandrene & limonene	99.5	88.1	93.8	110.1	157.8	134.0	82.9	82.3	82.6
cis-β-ocimene	19.8	19.3	19.6	19.9	28.1	24.0	15.2	15.3	15.2
terpinolene	77.6	76.0	76.8	81.4	111.6	96.5	62.1	65.0	63.5
β-caryophyllene	104	123	113	84	118	101	88	86	87
humulene	28.2	34.9	31.6	22.8	33.0	27.9	23.1	22.8	22.9
germacrene D	31.7	34.4	33.1	28.0	39.2	33.6	51.4	51.7	51.5
bicyclogermacrene	6.8	3.5	5.2	7.7	10.3	9.0	51.4	51.7	51.5
carylophyllene oxide	15.2	16.9	16.0	12.5	17.2	14.8	21.0	21.5	21.2
polyanthic acid methyl ester	143	167	155	126	164	145	141	193	167
hardwickic acid methyl ester	1368	1592	1480	1233	1593	1413	1372	1860	1616
20-oxo-hardwickic acid methyl ester	141	167	154	84	99	92	113	158	136

Table 3.1.ix. %Yields of Components from Blackcurrant Buds Using Varying Amounts of Propylene Glycol in the Extracting Solvent.

The inclusion of propylene glycol in the proportions trialed, produced unacceptable extracts as it could not be removed by RVE and the residual solvent separated from the blackcurrant extract. However, it should be noted that over a period of more than 2 years, the products from this experiment retained a potency far beyond that of extracts produced using standard extraction protocols, indicating that propylene glycol may

contribute to preserving aromatic qualities. This evaluation was based purely on an organoleptic appraisal by assessors well versed in the nuances of blackcurrant aroma (no data available).

3.1.7. Extraction of Blackcurrant Buds Using the Laboratory Based Method with the Inclusion of the Antioxidant BHA

To further protect labile chemicals from oxidative processes in the extraction method developed whereby buds are initially steeped in ethanol prior to partitioning into non-polar solvents, an antioxidant (BHA) was included. Although the concrete and the marc were dried down, analysed and stored separately the yields relative to the weight of buds extracted were summed and are presented in table 3.1.x.

%yield from buds (DMB)	no antioxidant			antioxidant		
	rep1	rep2	**mean**	rep1	rep2	**mean**
concrete yield	6.4	5.6	**6.0**	6.4	7.1	**6.8**
volatiles	0.8	0.6	**0.7**	0.9	0.9	**0.9**
acids	2.3	2.2	**2.3**	2.5	2.6	**2.5**
extract not amenable to GC	48.9	45.3	**47.1**	48.2	46.6	**47.4**
%yield of component x 10^{-3} (DMB)						
α-thujene	8	7	**7**	8	8	**8**
α-pinene	8	3	**5**	5	7	**6**
sabinene	117	72	**95**	97	115	**106**
myrcene	18	12	**15**	16	18	**17**
δ-3-carene	126	80	**103**	108	128	**118**
β-phellandrene & limonene	103	72	**88**	94	106	**100**
cis-β-ocimene	23	17	**20**	21	23	**22**
terpinolene	97	73	**85**	93	100	**97**
β-caryophyllene	149	111	**130**	145	151	**148**
humulene	40	35	**37**	40	41	**41**
germacrene D	50	43	**47**	51	52	**52**
bicyclogermacrene	14	11	**13**	14	15	**15**
carylophyllene oxide	17	16	**16**	18	18	**18**
polyanthic acid methyl ester	190	183	**186**	206	212	**209**
hardwickic acid methyl ester	1829	1758	**1793**	1964	2036	**2000**
20-oxo-hardwickic acid methyl ester	176	172	**174**	193	188	**190**

Table 3.1.x. %Yield of Components from Buds (DMB) with Antioxidant vs No Antioxidant in Extracting Solvents.

The inclusion of BHA in the extracting solvent did not appear to improve the yield of volatile components. The GC analyses of the extracts were repeated over three months to determine if the antioxidant had an effect during extract storage. Extracts (excluding the marc) were analysed on three occasions, 0, 23 and 51 days after extraction. GC FID was undertaken on each day of analyses, however, GC FPD was only performed on the first and final day of storage. Results are presented as the % composition of each component relative to the weight of extract.

component	days	0	mean	26	mean	51	mean	0	mean	26	mean	51	mean
		no antioxidant						antioxidant					
thujene	rep1	0.15		0.14		0.17		0.14		0.12		0.11	
	rep2	0.13	**0.14**	0.13	**0.13**	0.16	**0.17**	0.13	**0.13**	0.13	**0.12**	0.14	**0.13**
α-pinene	rep1	0.16		0.12		0.05		0.08		0.05		0.02	
	rep2	0.07	**0.12**	0.05	**0.09**	0.02	**0.04**	0.15	**0.12**	0.12	**0.09**	0.06	**0.04**
sabinene	rep1	2.08		1.76		1.17		1.42		1.06		0.63	
	rep2	1.35	**1.72**	1.09	**1.42**	0.67	**0.92**	2.10	**1.76**	1.82	**1.44**	1.30	**0.96**
myrcene	rep1	0.34		0.31		0.21		0.26		0.20		0.13	
	rep2	0.24	**0.29**	0.20	**0.25**	0.12	**0.17**	0.34	**0.30**	0.31	**0.26**	0.24	**0.18**
δ-3-carene	rep1	2.49		2.21		1.55		1.81		1.42		0.91	
	rep2	1.67	**2.08**	1.43	**1.82**	0.86	**1.20**	2.45	**2.13**	2.21	**1.82**	1.71	**1.31**
β-phellandrene	rep1	2.11		1.95		1.48		1.68		1.37		0.99	
& limonene	rep2	1.55	**1.83**	1.40	**1.68**	0.96	**1.22**	2.03	**1.86**	1.89	**1.63**	1.56	**1.28**
cis-β-ocimene	rep1	0.44		0.42		0.33		0.37		0.31		0.24	
	rep2	0.34	**0.39**	0.32	**0.37**	0.24	**0.28**	0.43	**0.40**	0.41	**0.36**	0.35	**0.30**
terpinolene	rep1	1.88		1.80		1.42		1.63		1.39		1.10	
	rep2	1.51	**1.70**	1.44	**1.62**	1.08	**1.25**	1.84	**1.73**	1.78	**1.58**	1.51	**1.31**
β-caryophyllene	rep1	2.70		2.44		2.34		2.62		2.21		2.14	
	rep2	2.20	**2.45**	2.30	**2.37**	2.27	**2.31**	2.41	**2.52**	2.32	**2.27**	2.32	**2.23**
humulene	rep1	0.67		0.04		0.62		0.63		1.88		0.57	
	rep2	0.61	**0.64**	0.04	**0.04**	0.61	**0.62**	0.64	**0.63**	1.59	**1.74**	0.63	**0.60**
germacrene D	rep1	0.85		0.79		0.67		0.82		0.73		0.66	
	rep2	0.79	**0.82**	0.75	**0.77**	0.66	**0.67**	0.84	**0.83**	0.79	**0.76**	0.72	**0.69**
bicyclogermacrene	rep1	0.23		0.18		0.12		0.23		0.18		0.13	
	rep2	0.22	**0.23**	0.18	**0.18**	0.12	**0.12**	0.26	**0.24**	0.21	**0.19**	0.15	**0.14**
carylophyllene	rep1	0.30		0.40		0.36		0.30		0.36		0.32	
oxide	rep2	0.31	**0.31**	0.42	**0.41**	0.39	**0.37**	0.29	**0.29**	0.36	**0.36**	0.33	**0.32**
polyanthic acid	rep1	2.86		3.07		2.76		2.91		3.08		2.72	
	rep2	3.04	**2.95**	3.31	**3.19**	3.12	**2.94**	2.95	**2.93**	3.16	**3.12**	2.84	**2.78**
hardwickic acid	rep1	28.7		30.1		27.0		28.8		29.4		29.4	
	rep2	30.3	**29.5**	32.4	**31.2**	30.4	**28.7**	29.0	**28.9**	30.2	**29.8**	28.7	**29.0**
20-oxo-	rep1	2.68		2.96		2.67		2.80		3.06		3.09	
hardwickic acid	rep2	2.93	**2.80**	3.35	**3.15**	3.19	**2.93**	2.54	**2.67**	2.82	**2.94**	2.73	**2.91**

Table 3.1.xi. %Yield of Components (mean ±SD) in Blackcurrant Extracts Produced With and Without Antioxidant Present in the Extracting Solvent Over Time (days)

The marc was not re-analysed so that the results listed in table 3.1.xi relate only to the extracts produced from the non-polar partitions separated from the ethanol extract. Only slight differences in the relative composition of the extracts produced with and without antioxidant were noted over time. Table 3.1.xii records the level of thiol detected in the preliminary extract produced with and without antioxidant.

	days			
	0	51	0	51
	no antioxidant		antioxidant	
	$mgkg^{-1}$ thiol	$mgkg^{-1}$ thiol	$mgkg^{-1}$ thiol	$mgkg^{-1}$ thiol
rep1	22.8	17.9	20.2	18.2
rep2	20.7	17.1	21.6	18.1
mean	**21.8**	**17.5**	**20.9**	**18.1**

Table 3.1.xii. Levels of Endogenous Thiol ($mgkg^{-1}$) in Blackcurrant Extract Produced With and Without Antioxidant.

These results may be more easily interpreted by relating the concentration of components over time relative to the amount originally detected. Table 3.1.xiii present results for the percentages of volatiles, acids and the endogenous thiol, 4-methoxy-2-methyl-2-butanethiol, remaining in blackcurrant extracts over time.

% remaining	storage time (days)	antioxidant	no antioxidant
volatiles	0	100	100
	26	92.1	91.3
	51	79.6	76.3
acids	0	100	100
	26	109.4	111.6
	51	110.5	114.2
thiol	0	100	100
	26	86.9	80.5

Table 3.1.xiii. % Composition of Volatiles, Acids and Thiols in Blackcurrant Concretes Over Time.

Also of note is that when the samples were removed from storage 5 years later and assessed organoleptically, the samples to which BHA had been added retained dramatically more potent blackcurrant aroma when compared to the samples to which no anti-oxidant had been added. In the extraction process the addition of antioxidant to the extracting solvent did not significantly improve the yield of most components. Subsequent to extraction the antioxidant appeared to retard the loss of volatiles in extracts. Another aspect highlighted by the results was the relatively low depletion rate of the thiol in all extracts produced. The ethanol based extraction protocol may serve to stabilise thiols. This aspect is further investigated in section 3.11.2.

3.1.8. Effects of Freezing Blackcurrant Buds in Ethanol.

The steeping of fresh buds in ethanol and storing them frozen has the potential to retard enzymatic activity and would have the added advantage that it also constitutes the first step in the extraction process developed in this study. Un-rolled and rolled buds were stored frozen in ethanol for a period of 2 months and compared to buds that had been immediately frozen un-rolled and without solvent. The yields and thiol levels recorded for extracts produced from buds stored using 3 methods are listed in table 3.1.xiv.

treatment (n=3)	thiol $mgkg^{-1}$ (DMB)	%yield $mgkg^{-1}$ (DMB)
un-rolled frozen in ethanol	526±56	6.1±0.2
rolled frozen in ethanol	408±17	6.4±0.4
frozen without ethanol	520±41	5.7±0.3

Table 3.1.xiv. Levels (mean ±SD) of Thiol and %Yield of Extracts from Blackcurrant Buds Frozen in Ethanol vs Buds Frozen Without Ethanol.

The quality and yields of extracts from buds that had been left intact and frozen in ethanol were slightly higher but did not result in sufficient improvements to warrant adoption of this method of storage.

3.1.9. Adaptation of the New Blackcurrant Extraction Method to Commercial Operations

In the previous sections a new blackcurrant bud extraction techniques had been developed using ethanol, which has been shown to deactivate degradative enzymes (Tressel, 1970) and penetrate plant material for efficient extraction of components. Additives such as antioxidants and propylene glycol were shown to not signicantly improve the recovery of quality components. Industry, historically, had used 5 % hexane in petroleum ether in direct extraction of the rolled buds. Using the fundamentals of this protocol, but including the ethanol as the preliminary extracting solvent, a product with a significantly altered aroma profile was presented to Industry for assessment. Industry showed much interest in the samples and the method by which they were produced were reviewed so as to facilitate adaptation to industrial processes. The preliminary laboratory based method developed by continuous improvement follows the steps described below;

Preliminary Laboratory Based Method

- Roll frozen blackcurrant buds and immediately immerse in 4 x w/v ethanol
- Agitated for 2 hours and decant solution
- Reduce ethanol volume by 50 % and add water at a ratio of 1.4 (w/v)
- Partitioned the extract solution into 4 x w/v of 5 % hexane in petroleum ether.
- Repeat partition 2 times
- Re-extract the buds in 400 mLs of 10 % ethyl acetate in 5 % hexane in petroleum ether
- Combine the all the petroleum ether based extracts and dry down by RVE.

In the following section four aspects were investigated that would result in protocols more suited to the established industrial application, the first of which was to reduce ethanol volumes. These were too high to be easily managed in the large-scale extracting drum at the factory and considering the increased expense of the solvent, experiments were conducted to determine the minimum ethanol volume required for efficient extraction of the buds (section 3.1.9.i). Indeed it was considered favourable if

the volumes of non-polar solvent could also be reduced. The effectiveness of the parition between ethanol and the 5 % hexane in petroleum ether, without the reduction in ethanol volume by RVE and without the addition of water, is reported in section 3.1.9.ii. Having established aspects that may be adapted to allow for industrial limitation, the extract produced using the modified method is compared to the extract produced using laboratory based method in section 3.1.9.iii. The yields relative to the buds originally extracted are determined at each step in the 2 extraction methods. Disparities in the extracting methods resulted in the initiation of experiments into the effect on extract yields of ultra-turrexing buds compared that from rolled buds not subject to ultra-turrexing (section 3.1.9.iv).

3.1.9.i. Reduction of Ethanol Volume from 1 to 4 (bud weight/solvent) to 2 to 1.
Industry limitations required that the initial volume of ethanol added to buds be reduced from 1:4 to 1:2. This was to be followed by 2 x 1:1 (w/v) ethanol extractions to compensate for the lower initial volume. This experiment was designed to determine the recovery of blackcurrant components from buds extracted with 1:2 ethanol : bud (w/v) initial extraction with 2 sequential 1:1 ethanol : bud (w/v) extractions. The extract yields and recovery of ethanol available for recycling were also recorded and are listed in table 3.1.xv.

ratio of bud weight : solvent volume	volume of ethanol (mL)	volume of filtrate recovered (mL)	%solvent recovered	weight of extract (g)	% yield
1 : 2	100	78	78	3.98	7.96
1 : 1	50	38	76	0.68	1.36
1 : 1	50	44	88	0.99	1.98
1 : 1	50	46	92	0.31	0.62

Table 3.1.xv. The Yield of Blackcurrant Extract and the Percentage Solvent Recovery from Sequential Ethanol Extractions of Buds.

The higher yield evident in the third 1:1 ethanol wash compared to the second wash may be explained. Buds are approximately 50 % water such that the first ethanol wash would remove some of this water. In the second 1:1 ethanol wash there would be less water in the buds as a result. When the third 1:1 ethanol extraction is undertaken it may be postulated that most of the water is removed in the first and second extraction so that this extracting solvent will be of lower polarity and may extract component not readily soluble in aqueous ethanol solutions. In all, the 1 wash of 2:1 ethanol : bud and 2 of 1:1 ethanol : bud washes appears to be sufficient to remove most of the ethanol soluble components, whilst any residual volatiles will most likely be recovered in the10 % ethyl acetate in 5 % hexane in petroleum ether extraction undertaken in the full method. The recovery of ethanol from the three extractions was 80 %.

Table 3.1.xvi records the yields of several of the components identified. Selections were made based on the confidence of identification based on retention time and pattern of elution and to include representatives of most types of component, for example monoterpenes, sesquiterpenes and acids. The percent volatiles are not shown because many of the peaks in the chromatogram could not be positively identified, as opposed to previous identifications based on non-polar extracts. The fourth extraction was not analysed by GC FID as there was insufficient recovery of oil.

component	ethanol added (w/v)		
	1:2	1:1	1:1
α-pinene	2.84 (72%)	0.44 (11%)	0.64 (16%)
sabinene	3.94 (78%)	0.59 (12%)	0.54 (11%)
β-caryophyllene	27.9 (67%)	6.4 (15%)	7.3 (18%)
humulene	9.3 (70%)	1.8 (14%)	2.2 (17%)
polyanthic acid	43.7 (77%)	7.7 (14%)	5.5 (10%)
hardwickic acid	390 (78%)	71 (14%)	42 (8%)

Table 3.1.xvi. Components ($mgkg^{-1}$) Extracted During Sequential Ethanol Extractions of Blackcurrant Buds.

The recovery of monoterpenes, as exemplified by α-pinene, was higher in the third extraction than in the second. The increases observed for these hydrocarbons, as opposed to the decreases in the recovery of the acids, which are more polar, further supports the postulation that the third ethanol extraction contains less water and is therefore less polar than the second ethanol extraction.

From this experiment it was proposed that whilst the total 3 washes of 1 x 1:2 and 2 x 1:1 would be optimal for complete extraction of ethanol soluble components, any components that may be left un-extracted following a 1 x 1:2 and a 1 x 1:1 wash would be recovered in the final back-wash of the extracted buds using 10 % ethyl acetate in 5 % hexane in petroleum ether.

3.1.9.ii. Exclusion of the Volume Reduction Step of the Ethanol Extract and the Effect of the Addition of Water on the Partitioning Between Polar and Non-polar Layers.

In the laboratory based method the volume of the ethanol was reduced using RVE. This is logistically complicated within the industrial context. This experiment investigated the effectiveness of the partitioning of the polar extract into 5 % hexane in petroleum ether when the volume of ethanol extract was not reduced. The effect of the

inclusion of water on the partitioning process was investigated. The process followed is simplified in the flowchart in figure 3.1.i.

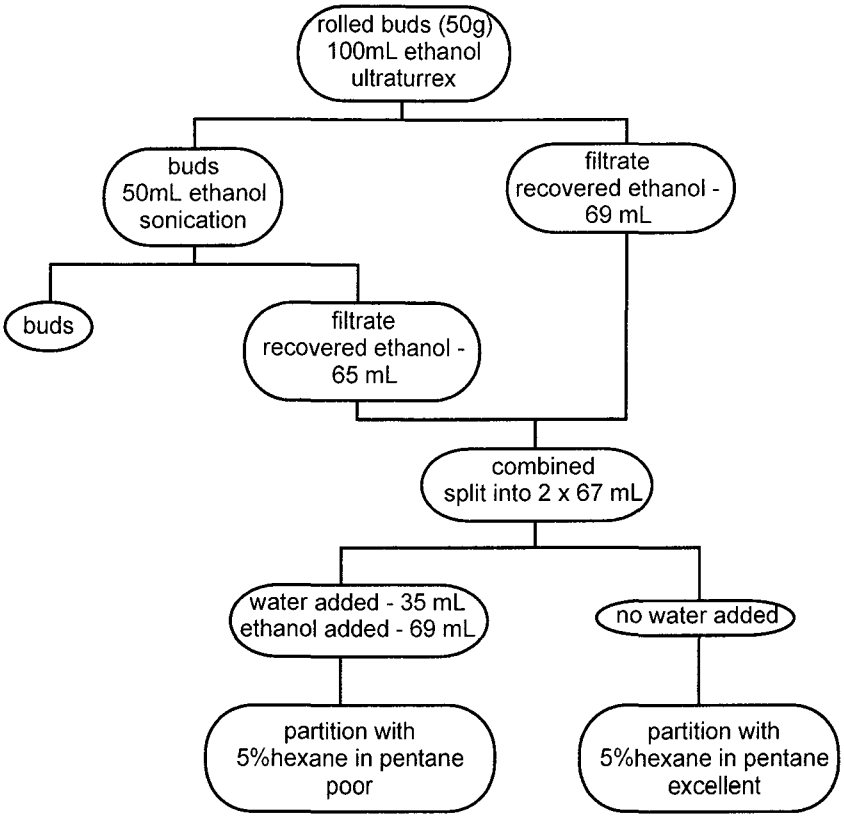


Figure 3.1.i. Summary of Procedures Undertaken to Determine the Effectiveness of an Aqueous Ethanol Partition.

In the procedures that formed part of the new laboratory based extraction method, the reduction of ethanol volume and the addition of water resulted in a well-defined partition when non-polar solvents were added. If the method is to be altered, maintaining the original volumes of ethanol but excluding the dry down of the ethanol extract by RVE, then water should not be added. This resulted in a poorly defined line of separation between the polar and non-polar solvents. However, with the proposed lowering of ethanol to be included in the modified method adapted for industry, a distinct partition formed without the addition of water.

3.1.9.iii. Comparison of the Laboratory Based Extraction Method to the Method Adapted for Commercial Operations.

The new extraction method to produce improved yield and quality of extracts from blackcurrant used a volume of 1:4 (w/v) of ethanol that was subsequently reduced in volume by 50 % by RVE. Water was then added prior to the partitioning of the polar extract against 5 % hexane in petroleum ether (method 1 detailed on following page). The adaptation of this method to make it more practical for use on an industrial scale required that the volume of ethanol be reduced, which in turn necessitated that water not be added prior to partitioning (method 2 detailed on following page). In addition the ethanol extract was partitioned against 3 x 75 mL of 5 % hexane in petroleum ether in method 2 compared to 3 x 400 mL in method 1. Finally the marc was extracted with 3 x 100 mL of 10 % ethyl acetate in 5 % hexane in petroleum ether in method 2 to replace the single 400 mL used in method 1. Buds were extracted using the two methods to assess if the new adaptations compromised the quality or yield of the final product. The volume of ethanol added to buds in method 1 proved to be a problem when added to the extraction bottles. As the vessels tumbled on the rotary drum the absence of air pocket in the near full vessels reduced the momentum of the fall of the buds within the solvent. Extraction was incomplete yielding only 0.92 % of extract compared to 4.81 % obtained from method 2. As such method 1 was repeated with buds ultra-turrexed in ethanol for several minutes. In the experiment subsequent to this one (section 3.1.9.iv) the effect that the inclusion of ultra-turrexing had on yield was assessed and the results were used to validate the direct comparison of the two extractions undertaken in this experiment.

EXTRACTION SUMMARIES

METHOD 1 *Full Laboratory Method*	**METHOD 2** *Modified for Industry*
100 g rolled buds + 400 mL ethanol	100 g rolled buds + 200 mL ethanol
Ultra-turrexed for 5 mins. - filter	Agitate for 2 hrs - filter
Reduce by 50 % with RVE	Add 100mL ethanol
Add 140 mL water	Agitate 30 mins
Partition 3 x 400 mL of 5 % hex. in pet. ether	Filter and combine with first extract
Combine partitions & evaporate	Partition 3 x 75mL 5 % hexane in pet. ether
Extract marc with 400mL 10 % ethyl acetate/5 %hexane in pet. ether Agitate 10 mins	Extract marc with 100mL 10 % ethyl acetate/5 % hexane in pet. ether Agitate 30 mins
Filter & dry down	Repeat with 2 x 100mL
	Filter partitions & marc extract
	Dry down

The effectiveness of the extraction method that had been adapted so as to be viable in commercial operations had been assessed by sub-sampling 0.5 mL from each extraction step. Table 3.1.xvii records the mg of monoterpenes, oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes and acids calculate to be present in each fracton.

solvent	ethanol		5% hex/pet.ether partition			10%EtOAC/5%hex/pet.ether		
wash	1	2	1	2	3	1	2	3
	mg	mg	mg	mg	mg	mg	mg	mg
ratio solvent:buds	(2:1)	(1:1)	(0.75:1)	(0.75:1)	(0.75:1)	(1:1)	(1:1)	(1:1)
monoterpenes	182	86	91	80	28	58	46	26
oxy. monoterpenes	39	20	16	12	5	13	9	5
sesquiterpenes	50	27	33	24	9	16	19	11
oxy. sesquiterpenes	10.0	6.4	3.0	4.2	6.1	2.0	1.4	1.1
acids	312	246	26	45	41	131	137	98

Table xvii. Recovery of components from each step of the modified extraction methodology (Method 2)

The results for the yields obtained using the two extraction methodologies are listed in table 3.1.xviii. It is apparent that the yield is reduced using the modified method 2. However this may be offset by the reduction in the yield of acids by almost 50 %. In addition method 1 produced concretes containing much higher levels of late eluting components of lower volatility and non-volatiles. Aroma impact assessment was not undertaken when concretes were fresh, however, after two years of storage an organoletic re-assessment of the oils indicated that concretes produced by both methods retained a strong blackcurrant quality.

%yields (wet weight)	etraction method	
	1-laboratory	2-modified
concrete	4.81	3.97
thiol ($mgkg^{-1}$)	6.2	5.8
volatiles in concrete	5.3	5.3
volatile yield from buds	0.26	0.21
α-thujene	0.004	0.003
α-pinene	0.000	0.000
sabinene	0.011	0.011
δ-3-carene	0.002	0.002
β-phellandrene & limonene	0.011	0.012
terpinolene	0.016	0.018
β-caryophyllene	0.069	0.063
humulene	0.020	0.018
germacrene D	0.007	0.007
carylophyllene oxide	0.007	0.005
polyanthic acid	0.084	0.039
hardwickic acid	0.673	0.373

Table 3.1.xviii. Effect of the Extraction Methods on Yields and Compositions of Blackcurrant Extract .

3.1.9.iv. Inter-relating the New Commercial Extraction to the Laboratory Based Method.

Results for the standard laboratory method (method 1) could not be directly compared to the new modified method (method 2) as method 1 included the application of an ultra-turrex for several minutes. Method 1 was repeated in conjunction with an

extraction identical in all respects but without the use of an ultra-turrex. In addition the importance of reducing the volume of the ethanol extract and the addition of water in terms of yield and aroma was assessed.

Table 3.1.xix lists the conditions employed and the yields of blackcurrant concrete for each extraction variation.

extraction method	code	extract %yield	%volatiles in GC fraction	%late eluting volatiles by GC
rolled/evaporated/water	2a	5.2	11.9	88.1
rolled/no evaporation/no water	2b	4.2	19.8	80.2
ultra-turrex/evapaporated./water	1a	4.5	11.2	88.8
ultra-turrex/no evaporation /no water	1b	4.2	22.4	77.6

Table 3.1.xix. Effect of Evaporation and Addition of Water on Yield of Concrete and Volatiles from Blackcurrant Buds.

The highest yield was obtained from buds that were rolled and extracted with the method which included the reduction in ethanol volume and with the addition of water prior to the partitioning into non-polar extracts (sample 2a). The concrete 1a, identified as having the most 'catty' quality was produced using identical protocols to 2a but without the use of an ultra-turrex. Sample 2a was nominated as having the next best quality. This indicated that the inclusion of the evaporation procedure and water addition was important to the aroma quality of blackcurrant concrete. These two concretes had a lower percentage of volatiles eluting prior to the internal standard, octadecane. The higher yield of late eluting components in the GC FID chromatogram was a characteristic common to all the oils produced using the ethanol based extraction. The effect of the volume reduction and water addition impacted more on extract yield than did the inclusion of ultra-turrexing.

Merging the results detailed in the preceding sections enabled a final extraction protocol to be presented to industry.

Blackcurrant Extraction Protocol - Commercial Adaptation

1. Roll frozen buds and immerse in 2:1 ethanol (v/w)
2. Agitate for 2 hours and filter (solvent recovery = 66 %)
3. Add another 1:1 ethanol and agitate a further 2 hours and filter (recovery = 110 %)
4. Add 0.75:1 5 % hexane in petroleum ether:original bud weight and mix
5. Allow phases to separate and draw of non-polar fraction

(Note; phase partition was very slow to form)

6. Repeat steps 6 and 7 twice more (total solvent recovery = 53.5 %)
7. Add 1:1 10 % ethyl acetate in 5 % hexane in petroleum ether to the bud residue from step 3
8. Agitate for 2 hours and filter (recovery = 42 % under vacuum)
9. Repeat steps 9 and 10 a further 2 times (recovery = 44 & 48 % consecutively)
10. Combine marc extract with the non-polar partition fraction and remove solvent

Lab. yield = 5.20 %
Thiol level = below detection level.

The filtrations in the method work-up were done under vacuum yet recovery of the solvents from the buds was often laboriously slow

The low levels of thiol were the combined result of poor quality buds (White Bud buds stored for over 2 months) and poor detector response by GC FPD on the day of analyses.

The parameters of the extraction methods that could have been tested for efficiency were limitless. This study, having been funded by Industry in conjunction with the Australian government, stayed within the constraints imposed by industry.

3.1.10. Stability of Endogenous Thiols in Blackcurrant Extracts.

In the following experiment the levels of 4-methoxy-2-methyl-2-butanethiols in commercially produced extracts are monitored over time. In the second section blackcurrant concretes are produced using the protocols developed in section 3.1.9 and

using the standard extraction methodology used in the industrial procedures. The stability of the 4-methoxy-2-methyl-2-butanethiol in these extracts during storage is investigated in addition to re-investigating the potential for antioxidants and humeticants to slow the depletion of thiols in the blackcurrant products post-extraction. This differs from the experiments detailed in sections 2.1.7 and 2.1.8, where propylene glycol and BHA were included in the extracting solvent to assess the potential of such additives to protect labile species through the extraction process. In this section the stability of labile volatiles in the final product, blackcurrant concrete, during storage is assessed.

3.1.10.i. The Rate of Depletion of Thiols in Commercially Produced Blackcurrant Extracts

Three commercially produced blackcurrant extracts (incubated and non-incubated, refer section 3.x.) were analysed by GC FPD over a period of a month to establish the rate of dissdipation of thiols in blackcurrant concrete. Table 3.1.xx lists the results.

days		Incubated White Bud $mgkg^{-1}$	Non-incubated high thiol $mgkg^{-1}$	Incubated high thiol $mgkg^{-1}$
0	rep1	45	63	54
	rep2	37	65	53
	mean	**41**	**64**	**53**
1	rep1	36	62	52
	rep2	40	57	44
	mean	**38**	**59**	**48**
8	rep1	27	43	31
	rep2	26	42	33
	mean	**27**	**43**	**32**
29	rep1	10	28	11
	rep2	12	29	5
	mean	**11**	**29**	**8**

Table 3.1.xx. Thiol Levels ($mgkg^{-1}$) in Commercially Produced Concrete Over 29 Days.

Results presented in table 3.1.xx show that as much as 85 % of endogenous thiol is lost from extracts stored for more than 1 month.

3.1.10.ii. Effect of Extraction Protocols and Antioxidants on the Depletion of Thiols in Blackcurrant Extracts.

Experimentation was instigated to determine whether the inclusion of antioxidants, humeticants or altered extraction protocols would reduce the depletion of endogenous thiol in blackcurrant extracts. Table 3.1.xxi records the yields obtained for each extraction method detailed in section 2.1.11.ii.

extraction method		% yield extract
standard procedure	rep1	3.6
	rep2	3.3
	mean	**3.5**
propylene glycol in standard procedure	rep1	3.8
	rep2	3.8
	mean	**3.8**
BHA in standard procedure	rep1	3.4
	rep2	3.5
	mean	**3.4**
ethanol based procedure	rep1	4.4
	rep2	4.1
	mean	**4.2**
propylene glycol in ethanol based	rep1	4.4
	rep2	4.7
	mean	**4.6**

Table 3.1.xxi. Yields of Concretes Produced Using Different Extraction Protocols.

The samples were analysed by GC FID and GC FPD on the day of extraction and at 1, 5, 7, 14, 21, 75 and 92 days (table 3.1.xxii).

extraction method		days							
		0	1	5	7	14	21	57	93
standard procedure	rep1	66	55	45	43	39	32	36	28
	rep2	59	58	44	41	32	38	36	28
	mean	**62**	**56**	**44**	**42**	**36**	**35**	**36**	**28**
propylene glycol -standard procedure	rep1	60	56	36	31	30	32	28	22
	rep2	54	46	40	25	28	30	27	21
	mean	**57**	**51**	**38**	**28**	**29**	**31**	**28**	**22**
BHA in standard procedure	rep1	54	56	32	32	28	36	28	32
	rep2	62	43	40	33	37	35	29	26
	mean	**58**	**49**	**36**	**32**	**32**	**35**	**29**	**29**
ethanol based procedure	rep1	sple lost		52	34	40	39	50	33
	rep2			50	37	43	40	57	37
	mean			**51**	**36**	**41**	**39**	**53**	**35**
propylene glycol in ethanol based	rep1	sple lost		49	37	50	39	53	26
	rep2			44	31	32	31	42	40
	mean			**47**	**34**	**41**	**35**	**48**	**33**

Table 3.1.xxii. Levels of Thiol ($mgkg^{-1}$) Detected in Blackcurrant Concretes Over Time.

The results from samples produced by ethanol extraction were not reliable as the extracts were not freely soluble in the solvent used for GC FPD analyses. The extraction process undertaken was not the optimised method and there were also inconsistencies in the methodology. The results are presented only as an indication that the rates of depletion of 4-methoxy-2-methyl-2-butanethiol may have been slower when ethanol is included in the extraction protocol.

The inclusion of antioxidant and propylene glycol did not reduce the loss of thiol when compared to the control (method 1 - extracts produced using the standard method). Aroma assessments of the concretes produced in methods 1 to 5 after several years of storage may reveal positive effects of the inclusion of additives over longer time periods.

Section 3.2. DORMANCY, FREEZING & INCUBATION

3.2.1. Chemical Composition of Blackcurrant Buds During Dormancy

Variations between the six clones selected for their high content of endogenous thiol (HTC) were not significant in terms of yield and composition of volatiles over the course of the dormant period. The data collected for the six clones at site 1 are presented as a mean in all the experiments to follow. Figure 3.2.i shows the dry weight of buds at sites 1 and 2 harvested monthly, from senescence, in mid Autumn (early May), through to bud burst in late winter (mid September). At the final sampling at site 1, 23 % of buds had burst. The degree of bud burst at site 2 on the final collection date was not recorded. The changes observed prior to bud burst, are fitted to a linear regression model with slopes determined to be 0.013 (r^2=0.7) and 0.019 (r^2=0.7) for cv. White Bud at sites 1 (control) and 2 (commercial) respectively and 0.028 (r^2=0.9) for the HTC (site 2). Bud burst at site 1 occurred earlier than that at site 2.

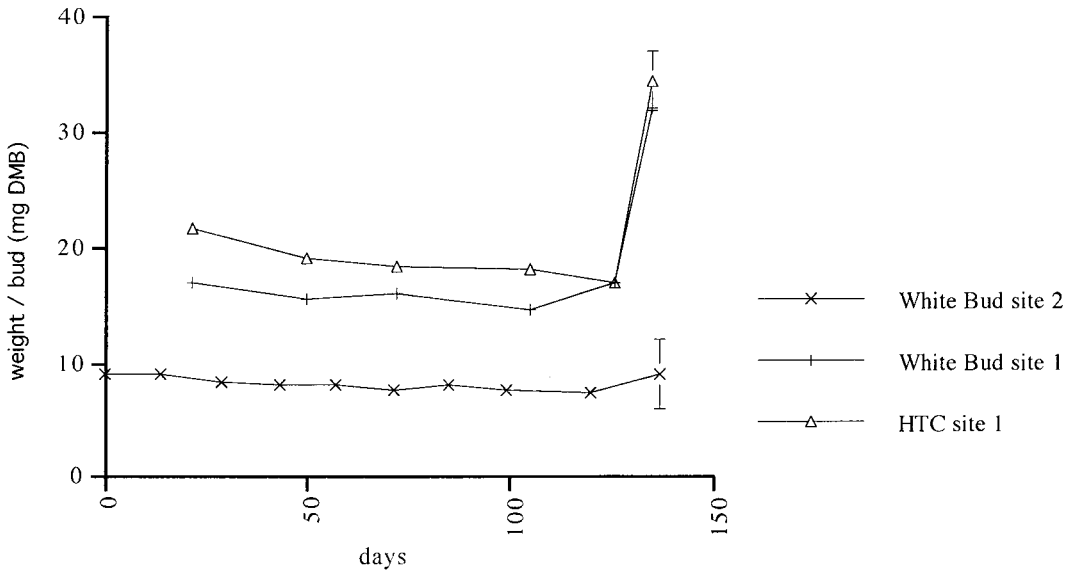


Figure 3.2.i. Weight / Bud During Dormancy

Concurrently, the yield of volatile components extracted from the buds decreased throughout the dormant period at both sites (figure 3.2.ii).

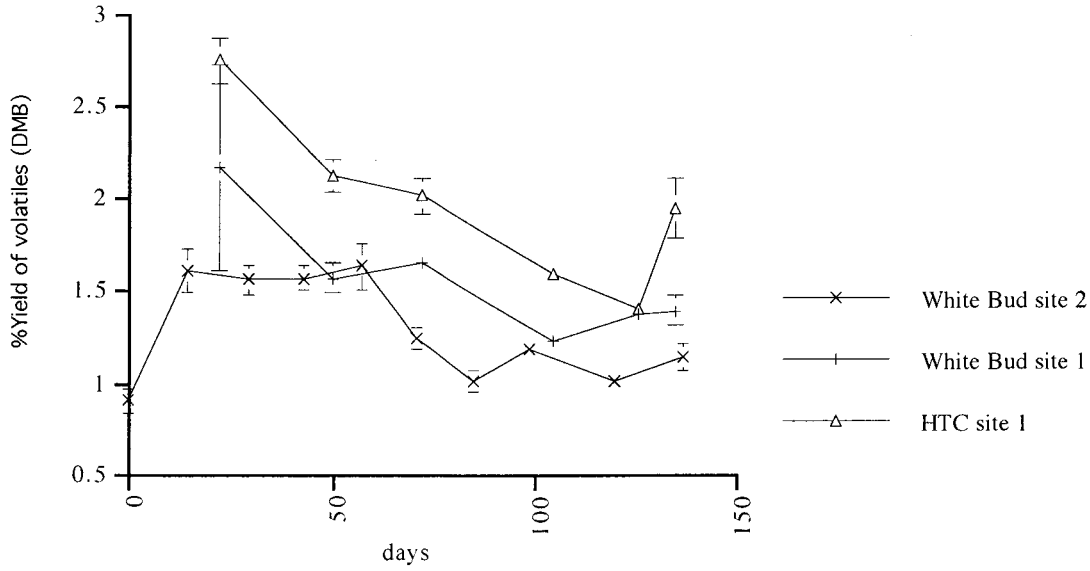


Figure 3.2.ii. %Yield of Volatile Components in Buds Throughout Dormancy.

The yield of volatiles relative to bud weight increased with the increase in bud weight at bud burst indicating that synthate is not only available for vegetative growth but for synthesis of volatiles.

Levels of thiol for each cultivar are given in figure 3.2.iii.

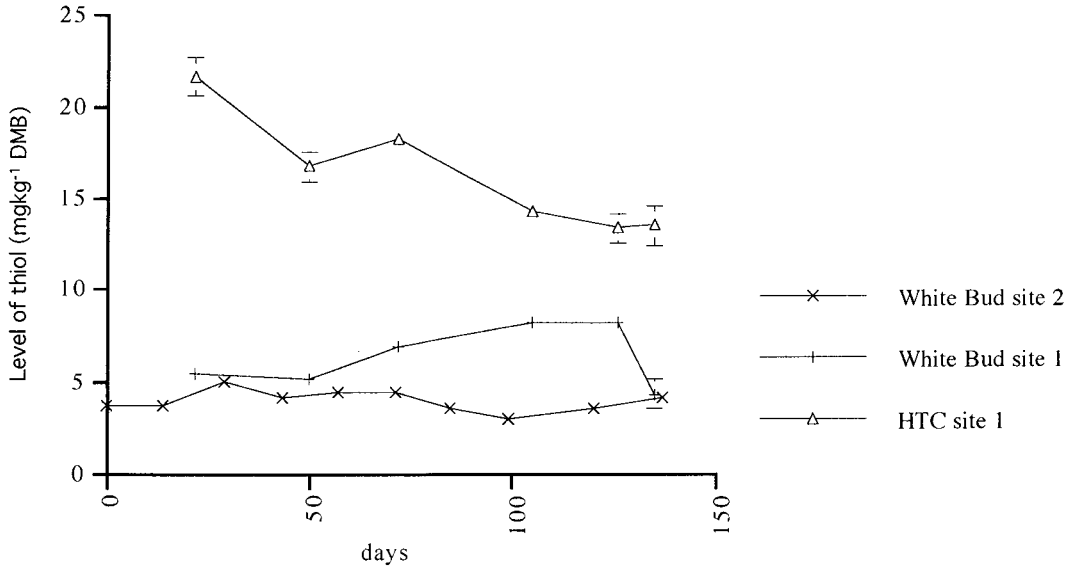


Figure 3.2.iii. Levels of 4-Methoxy-2-methyl-2-butanethiol in Blackcurrant Buds Throughout Dormancy.

In the HTC the level of endogenous thiols decreased from 22 ± 1 $mgkg^{-1}$ to 14 ± 1 $mgkg^{-1}$ throughout dormancy. In contrast, the level in cv. White Bud increased from 5.4 ± 0.3 $mgkg^{-1}$ to 8.2 ± 0.5 $mgkg^{-1}$ at site 1 but remained relatively constant at site 2.

At site 1 the loss of the 4-methoxy-2-methyl-2-butanethiol was consistent with the bud weight increase. The maintenance of levels observed at site 2 and in the HTC at site 1 indicated that synthesis of the thiol offset dilution due to growth.

Other notable differences in bud extract composition between the HTC and cv. White Bud were the levels of sabinene and bicyclogermacrene as illustrated in figures 3.2.iv and 3.2.v. Though higher in the HTC, both these parameters significantly decreased over the period of dormancy, a decrease which may be described by a linear regression with a slope of -6 x 10^{-3} (r^2=0.96) and -3 x 10^{-4} (r^2 = 0.87) for sabinene and bicyclogermacrene respectively. This rate of decrease is not repeated in cv. White Bud with slopes describing poorly defined linear regression of -1 x 10^{-3} (r^2 = 0.4) and -0.6 x 10^{4} (r^2 = 0.3) for sabinene and bicylcogermacrene respectively

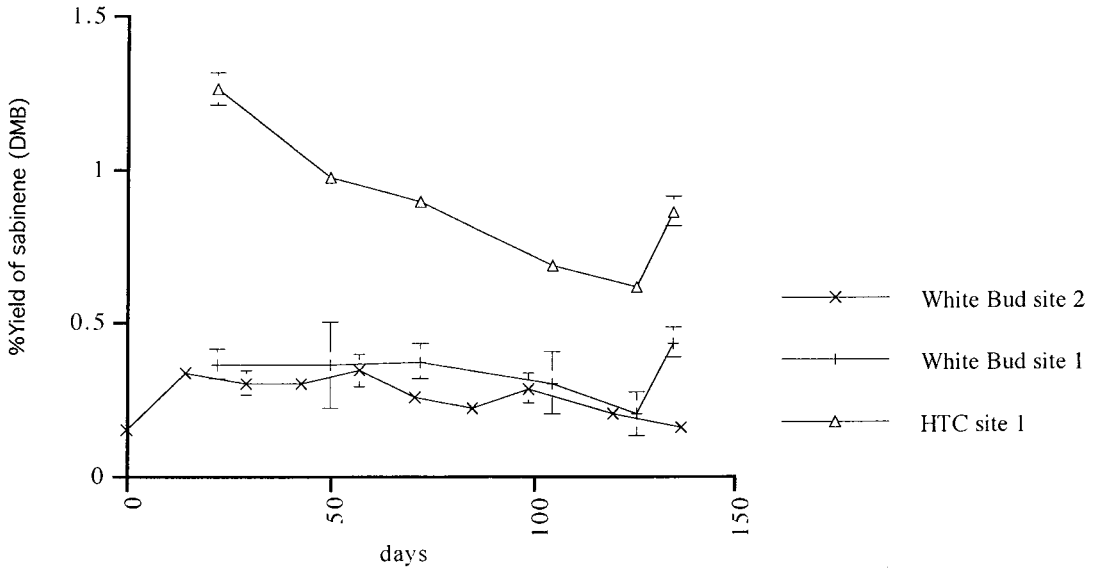


Figure 3.2.iv. %Yield of Sabinene in Blackcurrant Buds Throughout Dormancy

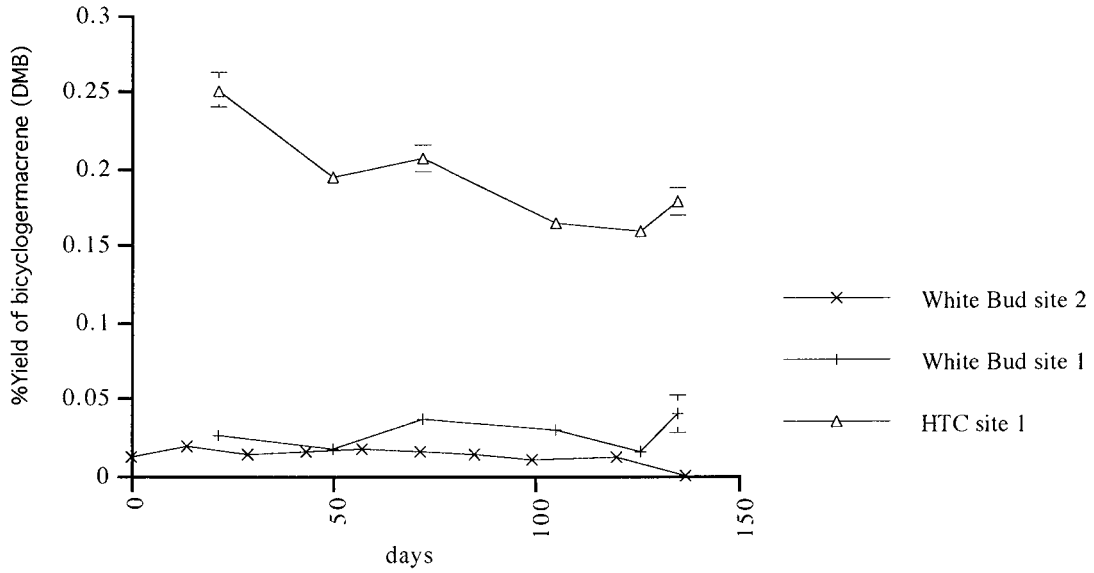


Figure 3.2.v. %Yield of Bicyclogermacrene in Blackcurrant Buds Throughout Dormancy.

Table 3.2.i. lists the changes in composition for other major components of blackcurrant extracts. The levels of myrcene and hardwickic acid are higher in the high thiol clonal stock. Over the period of dormancy the mean concentration levels at site 1 are 1.4 fold higher for both components.

days	%yield of component DMB x 10^{-3}					
	mycrene	δ-3-carene	β-phellandrene & limonene	terpinolene	β-caryophyllene	hardwickic acid
cv White Bud (site 2) (n=4)						
0	25±4	202±47	153±47	115±27	93±20	1015±239
14	48±11	343±64	243±64	194±36	133±17	384±101
29	45±8	369±58	268±54	194±30	128±18	263±36
43	46±6	366±61	266±53	195±32	141±20	462±84
57	48±12	368±61	264±58	194±42	137±33	474±84
71	35±6	265±46	192±52	146±26	125±19	387±56
85	27±6	212±46	146±46	113±25	104±17	395±90
99	32±4	247±36	162±58	125±19	110±8	466±49
120	27±3	219±35	157±47	110±19	105±10	556±136
137	31±4	293±39	217±31	138±19	119±10	646±57
cv White Bud (site 1) (n=10)						
22	62±21	542±214	400±173	298±119	159±51	1271±403
51	46.2±0.3	359±95	248±128	196±49	117±8	1265±306
80	47.1±0.4	386±52	262±61	202±26	126±11	1281±291
108	16±3	261±35	168±63	135±16	105±7	1006±5
139	37±1	332±39	253±40	167±12	117±10	2346±303
148	40±2	292±51	157±61	147±27	147±27	2103±112
HTC (site 1) (n=12)						
22	82±12	470±69	84±12	246±36	111±17	2738±405
51	63±9	350±30	69±10	182±27	84±11	1820±189
80	58±9	330±30	65±10	167±27	99±13	1797±239
108	46±4	260±30	61±6	132±10	78±6	1545±110
139	40±2	220±30	46±3	106±7	71±4	2883±278
148	53±12	320±20	56±12	151±33	151±33	2956±462

Table 3.2.i. %Yields of Components of Blackcurrant Bud (DMB) Throughout Dormancy for cv White Bud (site 2), and from cv. White Bud and HTC (site 1).

3.2.2. Effect of Freezing of Commercially Harvested Buds

The effects of freezing, and storage at -18°C on the thiol content and the terpene profile over time were investigated. Figure 3.2.vi. shows the effect of freezing on thiol content and %volatiles over 600 hours. Inset in figure 3.2.vi. is graph showing the

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change in volatile and thiol content over the course of 26 weeks. The results are expressed as the percentage yield relative to dry weight of buds extracted.

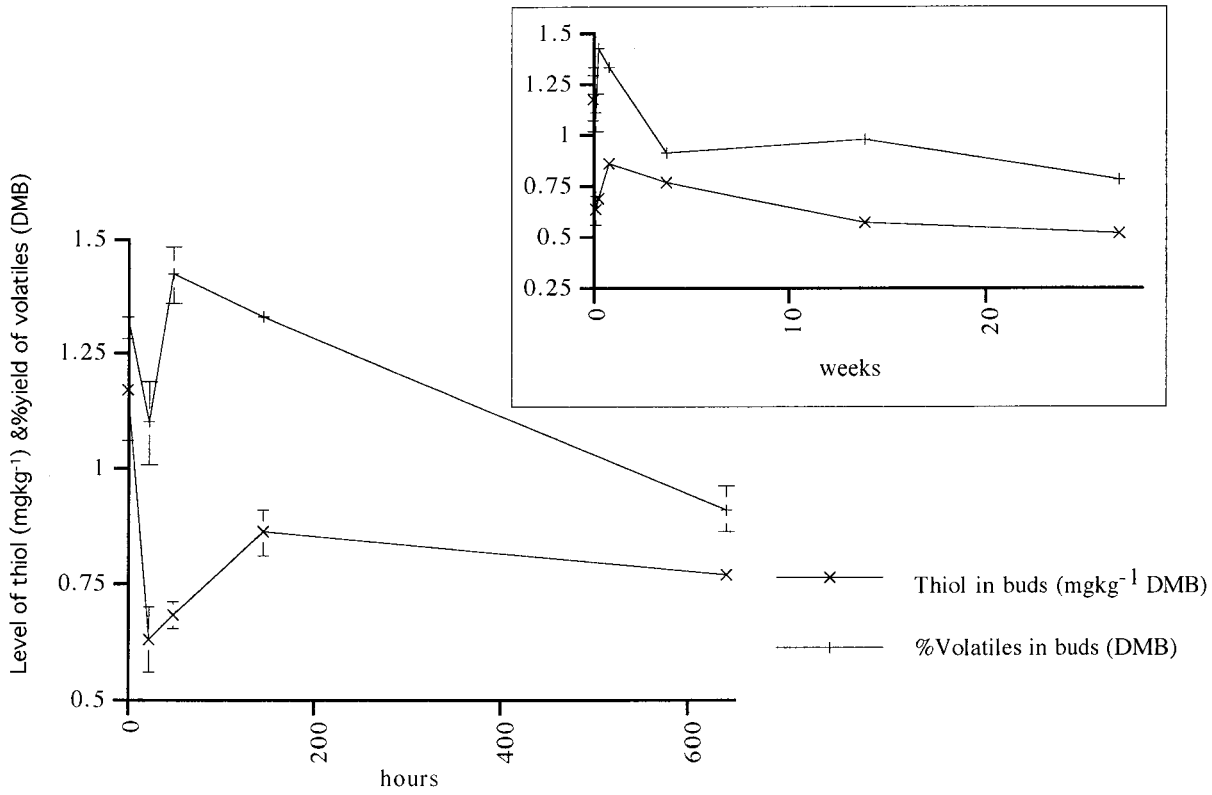


Figure 3.2.vi. Effect of Freezing on the Level of Thiol and Percentage Volatiles During Storage of cv. White Bud Buds. Inset -Long Term Effects.

Freezing of mechanically harvested buds resulted in a 54 % loss of thiols within 24 hours. However, continued storage at -18°C over a further 144 hours resulted in a small rise in thiol content so that the concentration reached 74 % of that recorded at time zero. Bud storage beyond that time, resulted in a decrease in thiol content to as low as 44 % of that detected in the fresh material. A similar pattern is observed for the volatiles. Freezing buds resulted in an initial decrease in the level of volatiles extracted, but continued storage at -18°C over 48 hours resulted in a increase in the level of volatiles extracted to above the concentration detected in fresh buds. Longer term storage resulted in a decline over 600 hours and thereafter volatiles were maintained at a constant level for 26 weeks.

Table 3.2.ii. records the levels of components extracted from frozen stored blackcurrant buds. Monoterpenes and sesquiterpenes account, in the greater part, to the initial increases observed in the volatiles (figure 3.2.vi) but these gradually decreased in concentration over the remaining months by 27 and 18 % respectively. By contrast, the levels of diterpene acids, hardwickic and polyanthic acid, remained relatively constant during the first month but after 3 months of storage at -18°C only 21 % of the acids that had been detected in the fresh material remained.

component (n=3)	%yield DMBx10^{-3}					
days	0	1	2	6	27	98
α-thujene	bdl	bdl	11±1	10±1	9+1	4±7
α-pinene	26±1	21±3	31±3	27±1	20±2	20±1
sabinene	508±6	451±65	585±41	562±19	379±29	377±19
mycrene	34.8±0.3	28±4	37±3	36±2	24±3	28±1
δ-3-carene	259±7	215±30	286±29	259±7	187±17	182±8
β-phell+lim	93±7	69±11	96±15	80±6	18±4	64±2
ocimene	28±1	22±4	28±3	26±2	8±3	23±1
terpinolene	120±4	96±15	131±13	119±4	94±9	87±4
β-caryophyllene	118±5	102±13	115±6	107±4	91±9	100±5
α-humulene	36±1	bdl	33±2	31±1	28±2	32±2
germacrene-D	55±3	32±4	49±3	47±2	34±16	45±3
bicyclogermacrene	16.3±0.5	46±7	15±1	13.4±0.5	13±2	9±8
caryophyllene oxide	bdl	14±2	bdl	7±6	7±2	bdl
polyanthic acid	168±11	143±22	138±5	121±3	148±13	bdl
hardwickic acid	1657±87	1435±206	1314±96	1193±23	1537±128	376±16

Table 3.2.ii. Levels of Components Extracted from Frozen and Stored Blackcurrant Buds.

3.2.3. Effect of Incubation on Thiol Content in cv White Bud and in HTC Buds (laboratory scale)

The incubation experiments are primarily designed to investigate the post-harvest synthesis of volatiles and the conditions that promote chemical processes of metabolism, catabolism, oxidation and degradation. Within this section the factors such as degree of damage incurred by the buds during harvest and the conditions of storage are investigated. The sections numbers assigned to experiments in 'Materials

and Methods', however, do not necessarily correspond to the section numbers reported in this results section. Instead results are reported and correlated as the objectives of the experiments undertaken are addressed. The objectives, then, were to investigate the following;-

1. effect of mechanical damage on the post-harvest synthesis of thiols.
2. effect of freezing damage on the post-harvest synthesis of thiols.
3. effect of mechanical damage on the post-harvest synthesis of volatiles.
4. effect of freezing damage on the post-harvest synthesis of volatiles

3.2.3.i. Effect of Mechanical Damage on the Post-harvest Synthesis of Thiols

Blackcurrant buds that were hand-cut from the cane had a higher thiol concentration than those machine-harvested (materials and methods section 2.3.ii). Thiol production was most rapid in fresh, hand-cut HTC buds incubated at 10°C in air with a steady increase from 4.6 $mgkg^{-1}$ at harvest to 10 $mgkg^{-1}$ after 50 hours (figure 3.2.iii.). This production was suppressed under a nitrogen atmosphere. The small rise of 4.6 to 6.5 $mgkg^{-1}$ recorded may be attributable to residual oxygen in the buds.

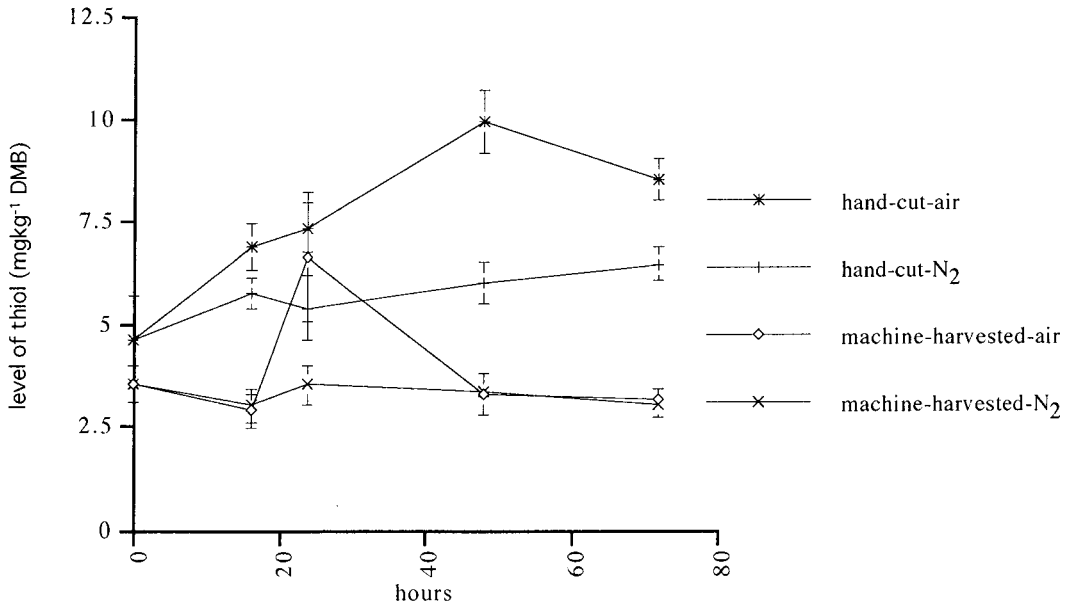


Figure 3.2.vii. Thiol Content in Fresh High Thiol Buds Incubated at 10°C.

A thiol loss of 18 % was recorded within 16 hours in mechanically harvested HTC buds. Only a small increase in thiol production was recorded over the 72 hour incubation thereafter. When cv. White Bud buds that had been machine-harvested on a commercial scale (materials and methods, section 2.2.3.i), were incubated whilst still fresh, 25 % of thiols were lost within 24 hours (figure 3.2.vii). In the longer incubation undertaken in this experiment the low level production of thiol in fresh machine harvested buds maximised within 72 hours but declined thereafter. Rolling fresh machine-harvested White Bud buds reduced thiol levels from 1.17 ± 0.05 to 0.69 ± 0.02 $mgkg^{-1}$ at time zero and within 24 hours of incubation at 10°C, no thiols were detectable. These results are not included in figure 3.2.vii. Similarly no thiols were detected in machine-harvested, frozen buds that were rolled and incubated.

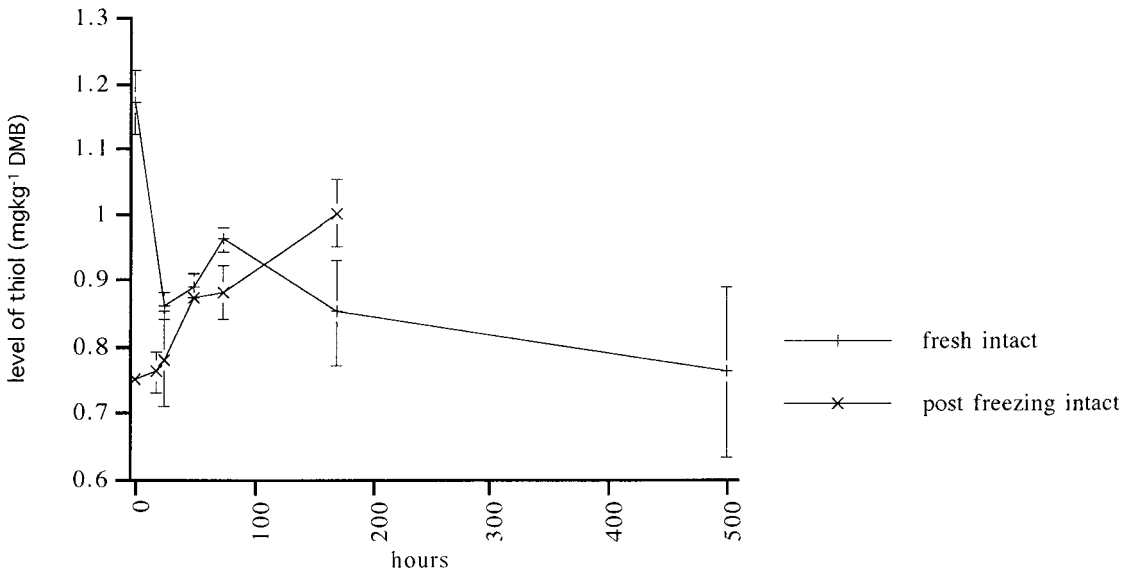


Figure 3.2.viii. Thiol Content in Un-rolled Commercial Buds Incubated at 10°C in Air.

In all post-harvest production of 4-methoxy-2-methyl-butanethiol was highest in incubated buds that had incurred minimal damage in the harvesting process. Fresh, machine-harvested buds had limited capacity for post-harvest thiol synthesis. This was evident in both HTC and White Bud buds.

3.2.3.ii. Effect of Freezing Damage on the Post-harvest Synthesis of Thiols.

The experiment using commercially produced White Bud buds detailed in section 2.2.3.i also investigated effects on post-harvest thiol production in machine-harvested buds that had been previously frozen at -18°C. In figure 3.2.vii, thiol content is reduced by 35 % when intact buds are frozen and defrosted prior to incubation at 10°C. Thereafter, thiols in frozen-thawed buds increase at a similar rate to fresh buds. Freezing did not appear to inhibit post-harvest synthesis in machine-harvested buds, on the contrary, synthesis appeared to be promoted by the freezing event to a degree not evident in fresh, machine-harvested buds. Endogenous thiols were not present in rolled, machine-harvested buds after freezing and there was no detectable post-harvest synthesis of 4-methoxy-2-methyl-2-butanethiol.

3.2.3.iii. Effect of Mechanical Damage on the Post-harvest Synthesis of Volatiles

The post-harvest production of volatiles in buds incubated at 10°C was greatest in hand-cut, fresh clonal buds (figure 3.2.viii) (Materials & Methods 2.2.3.ii.).

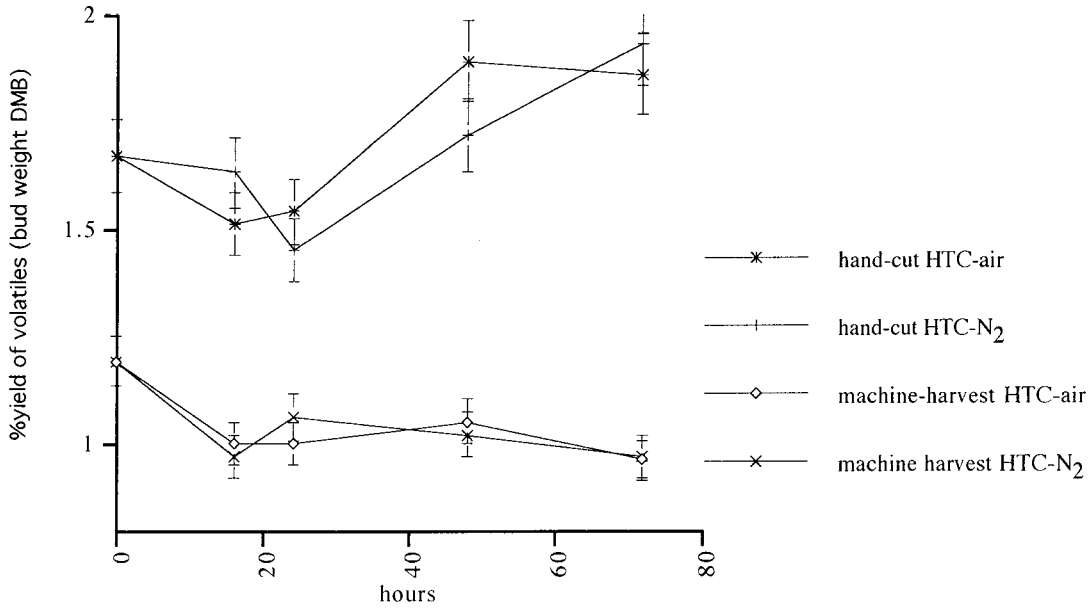


Figure 3.2.ix. %Volatiles in Fresh HTC Buds Incubated at 10°C.

Although 13 % of volatiles were lost within 16 hours, a 11 % increase above levels detected at the beginning of the incubation were recorded after 48 hours and levels

were maintained up until the end of the incubation at 72 hours. A nitrogen atmosphere only had a minimal effect on the suppression of volatile production. The levels of volatiles decreased by 19 % within 16 hours in buds which had been damaged by mechanical harvesting and levels had not increased at the end of the incubation at 72 hours.

Volatile yields for machine-harvested White Bud buds incubated prior to freezing and after freezing are given in figure 3.2.x (Materials & Methods 2.2.3.i).

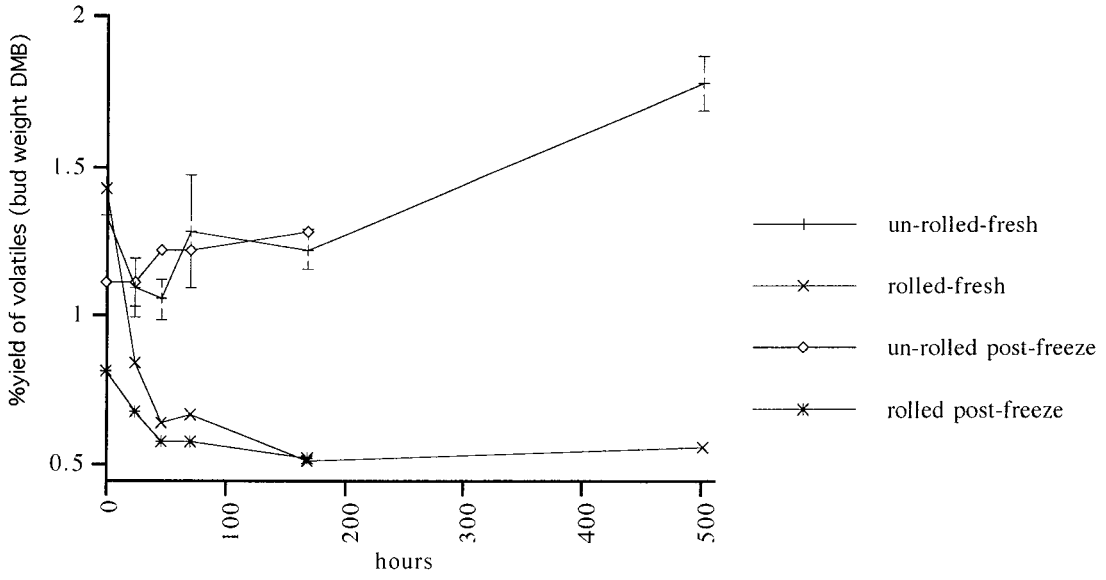


Figure 3.2.x. %Volatiles in commercial cv. White Bud buds incubated at 10°C in air.

Incubation increased the levels of volatiles in both fresh machine-harvested buds and buds frozen prior to treatment after 72 hours. Fresh un-rolled buds produced an increase in volatiles of up to 1.7 fold over 500 hours of incubation at 10°C. This rate of increase was closely mirrored in un-rolled, freeze-thawed buds for the 72 hour incubation. When mechanical damage was increased by processing buds through a roller, volatiles were depleted and post-harvest production retarded. In fresh buds that had been rolled, 45 % of volatiles were lost within 48 hours and no recovery was evident over 500 hours of post-harvest incubation. This dramatic depletion is also seen in buds frozen for 84 days before rolling and incubation.

Mechanical damage to buds incurred by the process of machine harvesting halted all post-harvest volatile synthesis in HTC and White Bud buds for the initial 72 hours of incubation. However, the rate of post-harvest volatile synthesis exceeded the rate of depletion in incubations undertaken for longer periods of time.

3.2.3.ix. Effect of Freezing Damage on the Post-harvest Synthesis of Volatiles

In figure 3.2.xi the level of volatiles produced in HTC buds, which have been frozen before incubation, is presented for machine-harvested and hand-cut buds.

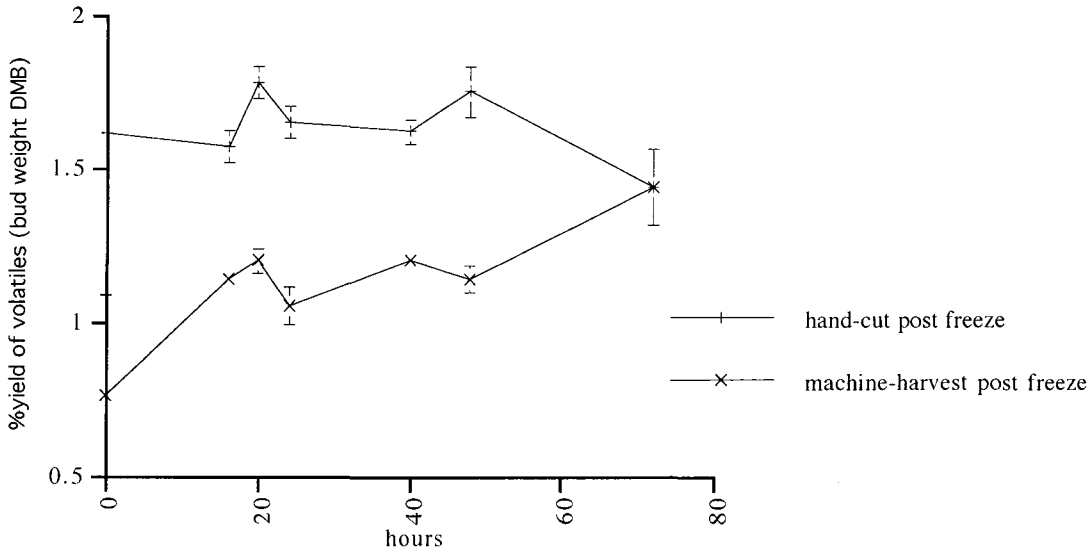


Figure 3.2.xi. %Volatiles in HTC Buds, Frozen Prior to Incubated at 10°C in Air.

Incubation of machine-harvested HTC buds that had been previously frozen resulted in a significant increase in the production of volatiles when compared to freeze-thawed, hand-cut buds and closely follows the trend already established in machine harvested White Bud buds which had been previously frozen (figure 3.2.x.). Post-harvest production of volatiles was not evident in previously frozen, hand-cut, HTC buds yet incubation of fresh, hand-cut HTC buds gave an overall 11 % increase in volatiles within 72 hours (figure 3.2.vii.). In summary, it appears that preservation of structure is required when HTC buds are incubated prior to freezing. However the effect of freezing these buds was to retard that synthesis. If those same buds are machine-

harvested and frozen, post-harvest volatile synthesis is evident. The structure of the bud is damaged by machine harvesting, rupturing cells. If ruptured cells promote synthesis in frozen buds, yet retards synthesis in fresh buds ,it may be proposed that the processes involved in fresh buds differ from those active in previously frozen buds.

Figure 3.2.xii. records the change in concentration of α-pinene, β-caryophyllene, caryophyllene oxide and hardwickic acid during the incubations. The pattern of post-harvest production of α-pinene was typical for all monoterpenes. Concentration levels decreased within the first 24 hours of all incubations at 10°C. In un-rolled buds, whether fresh or previously frozen, the levels of α-pinene increased or remained constant after 24 hours, whereas levels continued to decline in both fresh and frozen, rolled buds.

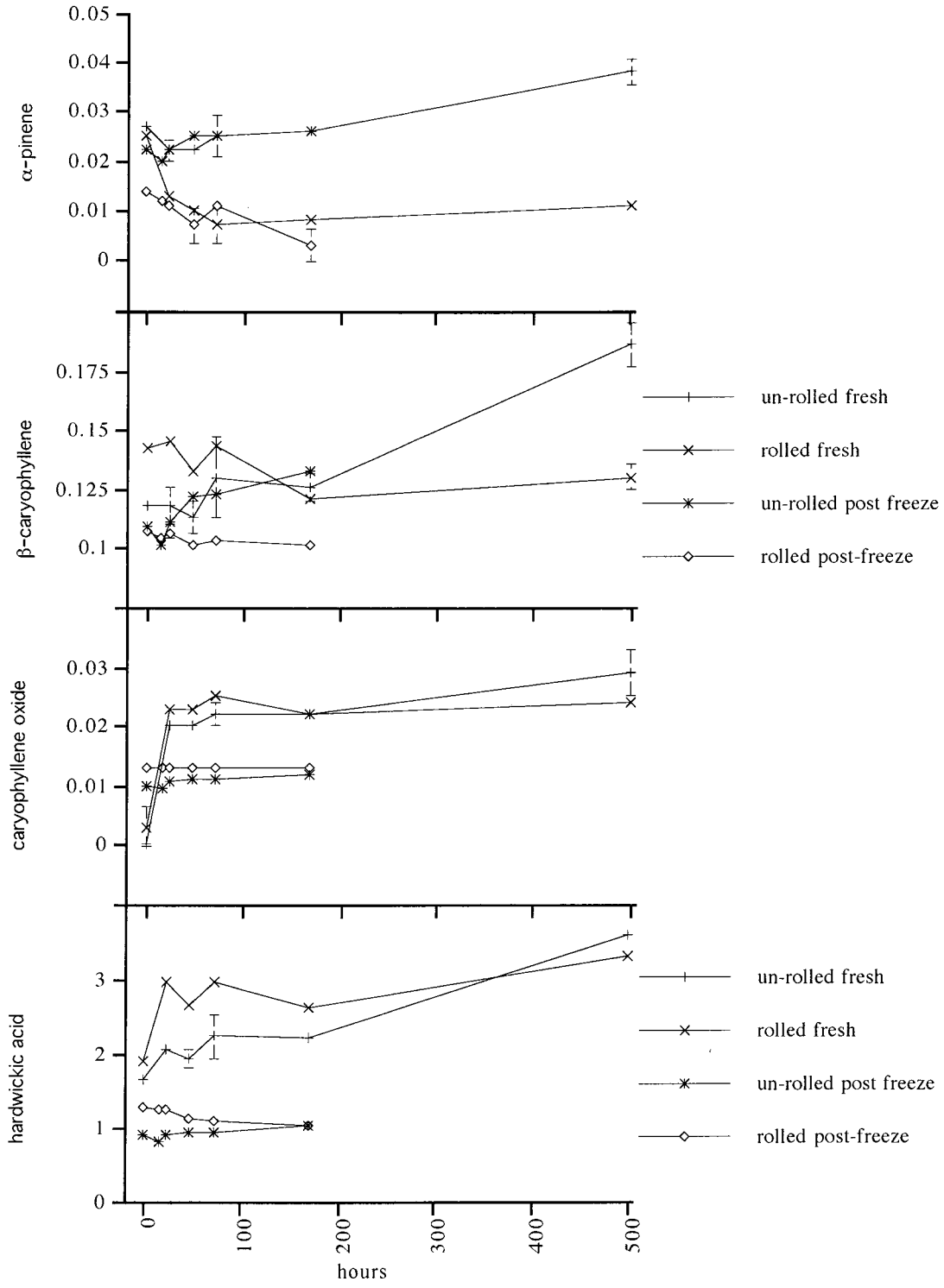


Figure 3.2.x. % Yield of α-Pinene, β-Caryophyllene, Caryophyllene oxide and Hardwickic Acid in Commercial Buds Incubated at 10°C.

The changes in concentration of β-caryophyllene in buds post-harvest were typical of most sesquiterpenes. Production of β-caryophyllene, as for α-pinene, continued in un-rolled buds, whether fresh or frozen. Despite the fact that the level of β-caryophyllene extracted from fresh rolled buds is 1.2 fold that extracted from fresh un-rolled at time zero, figure 3.2.xii indicates that all post-harvest synthesis of β-caryophyllene ceased in rolled buds, whether fresh or frozen. The decrease in sesquiterpene concentration in rolled buds occurred at a slower rate than that recorded for monoterpenes over the incubation period. In contrast, higher levels of oxygenated sesquiterpenes and acids were extracted from rolled buds at the beginning of the incubations, although similar recoveries of caryophyllene oxide from fresh rolled and un-rolled buds were recorded. For components such as caryophyllene oxide and hardwickic acid, the freezing of buds had a more pronounced effect on post-harvest synthesis than did rolling. Freezing deactivated all processes of synthesis and dissipation of oxygenated sesquiterpenes and acids.

3.2.4. Pilot-scale Incubation of Blackcurrant Buds for Improved Extract Quality and Yield.

Only solvated extracts have been produced in harvest and incubation experiment reported to date. This has precluded full assessments to quantify yield and quality of extracts. The larger scale experiments described in Materials & Methods 2.2.4 provided sufficient material to produce viable quantities of extract to allow for calculations of yields and for a full aroma assessment. The results for the pilot-scale harvest and incubation experiments are presented in the following tables. The extractions of samples harvested in July 2001 and incubated for 72 and 190 hours (samples 8 and 9) were extracted with re-cycled solvent so that any conclusions drawn are tentative.

Yield of oils and components

The mean weight per bud (DMB) did not change appreciably through the period of dormancy. Table 3.2.v. lists the harvest dates in 2001 and 2002.

	harvest year 2001			
days	0	28	63	87
date	28-May-01	25-Jun-01	30-Jul-01	23-Aug-01
	harvest year 2002			
days	0	28	63	No harvest
date	27 May 02	24 June 02	29 July 02	

Table 3.2.v. The Dates on Which Harvests Occurred in the Incubation Trials in 2001 and 2002.

The yields for 36 parameters were entered into a SAS database. For each dependent variable the effect of block, incubation hours, month and the inter-reaction between month and incubation hours were tested for the years 2001 and 2002. The P-values are listed in the appendix in tables A1 and A2. Only interactions with P-values below 0.05 are considered significant.

In tables 3.2.vi, 3.2.vii, 3.2.viii and 3.2.ix the yields (DMB) of the components from blackcurrant buds are presented in the harvest time and incubation trials in the years 2001 and 2002.

month (n=4)	harvest year 2001					
		%yield DMB		mgkg^{-1}DMB (x10^{-3})	%yield DMB (x10^{-3})	
	incubation hours	oil	volatiles	thiol	α-thujene	α-pinene
May	0	8.0±0.3	1.8±0.1	341±38	10.7±0.2	51±6
	69	7.9±0.2	1.8±0.1	201±34	10.3±0.6	42±4
	189	8.4±0.2	1.7±01	274±40	10.6±0.4	45±5
June	0	7.8±0.2	1.85±0.06	203±40	11.0±0.5	49±5
	69	7.6±0.3	1.9±0.1	142±29	11.0±0.8	46±5
	189	7.0±0.3	1.58±0.09	197±26	9.7±0.5	40±3
July	0	8.3±0.2	1.85±0.05	135±58	11.9±0.6	49±2
	69	9.5±0.4	3.3±0.3	620±58	18±1	80±5
	189	10.0±0.3	3.2±0.2	788±91	17.8±0.9	82±7
August	0	8.7±0.1	2.18±0.05	197±4	17±1	56±2
	69	8.47±0.03	2.00±0.09	376±22	17±1	53±3
	189	8.3±0.1	1.78±0.06	149±19	15±1	44±2
	harvest year 2002					
May	0	7.43±0.06	1.44±0.07	61±6	7.0±0.7	32±1
	69	7.3±0.3	1.3±0.1	101±16	8±2	26±2
	189	7.7±0.2	1.3±0.1	128±9	10±2	30±2
June	0	7.0±0.5	2.3±0.4	121±28	12±1	70±13
	69	6.9±0.4	3.1±0.6	181±322	15±1	104±22
	189	6.8±0.4	1.8±0.3	101±20	11±2	50±8
July	0	6.3±0.2	1.30±0.04	155±8	7.6±0.2	32.4±0.7
	69	6.5±0.1	1.23±0.05	83±3	11±1	34±3
	189	6.4±0.2	1.20±0.04	204±9	8.9±0.3	29±1

Table 3.2.vi. The %Yields of Components from Blackcurrant Buds in Harvest and Incubation Trials Conducted in the Years 2001 and 2002.

Tables A1 and A2 in the appendix confirm that the month of harvest had a significant effect on oil yields and recoveries of all components. The oil yield was increased when buds were incubated prior to extraction in July 2001 harvest year yet this increase did not occur in incubations undertaken in 2002. The levels of 4-methoxy-2-methyl-2-butanethiol increased when buds were harvested and incubated after June in 2001, whilst buds harvested as early as May benefited by incubation in 2002. The effectiveness of incubation in 2002 in increasing thiol yields, even in early harvests,

may be in part due to seasonal effects and this aspect is discussed more fully in section 4.2.4.

month (n=4)	harvest year 2001					
		%yield (x10^{-3}) DMB				
	time	sabinene	δ–3-carene	β-phellandrene & limonene	ocimene	terpinolene
May	0	340±14	379±31	251±27	33±3	216±17
	69	326±19	350±30	232±29	31±3	209±19
	189	334±12	342±33	221±29	29±3	194±18
June	0	345±10	386±30	260±26	33±3	224±17
	69	339±23	380±36	257±27	33±4	226±20
	189	281±15	318±20	213±17	27±2	187±11
July	0	323±17	389±10	267±11	32±1	222±6
	69	898±70	809±73	482±43	56±4	325±23
	189	849±44	763±46	448±28	54±3	308±16
August	0	482±22	443±12	281±9	36±1	242±6
	69	422±27	394±17	250±11	32±2	218±9
	189	375±19	348±13	219±12	28±1	191±7
	harvest year 2002					
May	0	276±52	286±9	187±14	44±1	182±7
	69	240±52	248±25	161±20	38±4	160±18
	189	253±52	269±18	174±9	39±3	166±12
June	0	442±64	553±100	368±80	77±14	307±59
	69	542±77	689±150	452±116	124±28	376±85
	189	320±81	381±68	246±45	51±10	209±39
July	0	224±5	264±8	180±8	38±1	153±5
	69	226±5	249±12	165±10	34±2	140±7
	189	199±5	228±12	152±10	32±2	130±7

Table 3.2.vii. The %Yields of Components from Blackcurrant Buds in Harvest and Incubation Trials Conducted in the Years 2001 and 2002.

The levels of monoterpenes and lower molecular weight components listed in table 3.2.vii show that bud levels remain fairly consistent when extracted without incubation up until bud burst in August in 2001. Only sabinene and δ-3-carene increased significantly immediately prior to bud burst. Incubation is most effective in July 2001.

The pattern is very different in 2002 when even without incubation the levels of components rose significantly in June.

month (n=4)	harvest year 2001					
	%yield DMB ($x10^{-3}$)					
	incubation hours	β-caryophllene	humulene	germacreneD	bicyclo-germacrene	caryophyllene oxide
May	0	165±5	47±1	65±3	25±1	20±2
	69	185±15	53±3	73±5	28±1	17±1
	189	179±11	52±2	70±4	26.6±0.6	16.0±0.8
June	0	173±12	48±3	67±4	23.6±0.8	22±1
	69	197±14	56±4	77±6	27±2	18±1
	189	171±7	49±2	66±3	23±1	15.5±0.8
July	0	179±8	51±2	68±1	23.0.8	17.9±0.6
	69	161±6	46±2	57.1±0.8	15.3±0.3	22±1
	189	150±7	42±1	55±2	17.9±0.7	15.8±0.7
August	0	197±6	58±2	77±2	25.8±0.8	20.2±0.9
	69	202±10	60±3	84±8	29±4	19.7±0.8
	189	185±10	56±3	72±2	24.1±0.8	19.0±0.8
	harvest year 2002					
May	0	146±8	43±4	62±4	24±1	14.0±0.3
	69	143±17	43±6	61±8	24±3	14±1
	189	142±15	42±5	60±7	23±2	14±1
June	0	171±29	45±8	65±11	25±4	16±2
	69	219±41	54±9	79±13	32±5	17±2
	189	143±25	39±7	55±10	21±4	16±1
July	0	132±3	36.4±0.4	51.7±0.7	18±2	15.6±0.4
	69	132±3	37±1	52±2	18±1	15.9±0.2
	189	131±5	37±1	51±2	18±1	15.8±0.4

Table 3.2.viii. The %Yields of Components from Blackcurrant Buds in Harvest and Incubation Trials Conducted in the Years 2001 and 2002.

Results presented in table 3.2.viii confirmed that many of the sesquiterpenes were not affected by incubation indicating that not only was post-harvest synthesis limited under the conditions trialed but also that this group of chemicals were stable in an aerobic environment whilst sequestered in blackcurrant buds.

The two un-identified chemicals listed in table 3.2.ix detected by GC FID are included in the results because it is of interest that their levels consistently rise through all incubations. This would suggest that they are by-products of the degradation of more labile chemicals or are a post-harvest synthesis by-product. Identifying these chemicals may allow for some suggestion as to what aroma impact these chemicals may confer on incubated products.

month (n=4)	harvest year 2001				
		%yield DMB ($x10^{-3}$)			
	incubation hours	unknown2	unknown 3	polyanthic acid	hardwickic acid
May	0	17±2	49±4	256±13	1877±81
	69	23±2	70±6	276±35	1932±256
	189	32±2	97±6	315±18	2416±87
June	0	9.8±0.3	30±4	232±25	1776±187
	69	22.9±0.7	42±2	356±24	2409±107
	189	20.3±0.5	48±1	314±17	2486±112
July	0	17±1	32±2	315±12	2709±48
	69	17±1	52+1	280±9	2335±58
	189	18±1	59±5	263±13	2093±116
August	0	14.0±0.4	45±1	337±10	2739±37
	69	19.6±0.5	66±1	358±13	2761±86
	189	22.9±0.7	76±2	343±11	2669±78
		harvest year 2002			
May	0	21±4	64±7	269±14	2262±146
	69	25±3	80±4	268±31	2289±273
	189	31±6	96±11	274±23	2349±209
June	0	15±2	43±4	197±35	1679±277
	69	17±1	46±9	214±40	1865±372
	189	25±3	73±13	236±39	2086±353
July	0	13±1	44±4	222±5	1887±47
	69	10±2	35±3	180±40	1488±334
	189	15±1	40±2	194±18	1625±173

Table 3.2.ix. The %Yields of Components from Blackcurrant Buds in Harvest and Incubation Trials Conducted in the Years 2001 and 2002.

Although acids contribute to the consistency of the product considered acceptable to the market, an excess is considered undesirable. Acid levels rose in some incubations undertaken in 2001, though not dramatically. In 2002 incubation had no effect on acid concentration in blackcurrant extract.

Aroma Assessment of the Extracts Produced in the Harvest and Incubation Trials

The pilot-scale harvests conducted in 2001 and 2002 presented the first opportunity for a complete aroma assessment. Ten judges participated in ranking the degree of cattiness for extracts produced in the trial in 2001 whilst thirteen judges were available in 2002. Table 3.2.x records the results for the 9 samples produced in 2002. The variance tables listed together for both years are recorded in table A3 of the appendix. Analysis of variance indicated there were significant differences in aroma impact in the 12 samples produced in 2001 whilst there was no significant difference between the aroma impact of the 9 samples analysed in 2002.

Table 3.2.x lists the extracts produced in both seasons in decreasing order of cattiness as assessed by the aroma assessment panel. The lower range conferred for the degree of cattiness in 2002 of 5.0 to 3.8 confirmed that there was little discernable difference in the extracts produced in the second trial year. Table 3.2.xi ranks the extracts in decreasing concentration of the level of 4-methoxy-2-methyl-2-butanethiol as detected by GC FPD.

	harvest year 2001											
extract#	8	10	11	9	12	7	5	6	4	1	2	3
month	July	Aug	Aug	July	Aug	July	June	June	June	May	May	May
hours	72	0	72	190	190	0	72	190	0	0	72	190
cattiness	7.6	7.3	6.9	6.8	6.7	5.8	5.4	5.2	5	4.7	4.5	4.1
	harvest year 2002											
extract#	8	4	7	5	9	2	6	1	3			
month	July	June	July	June	July	May	June	May	May			
hours	72	0	0	72	168	72	168	0	168			
cattiness	5.0	4.8	4.6	4.5	4.3	4.2	4.2	3.8	3.8			

Table 3.2.x. The Extracts Ordered from Highest Degree of Cattiness to the Lowest as Determined by the Aroma Assessment Panels in the Harvest and Incubation Trials Conducted in 2001 & 2002.

	harvest year 2001											
extract	9	8	11	1	3	4	2	6	10	12	5	7
month	July	July	Aug	May	May	June	May	June	Aug	Aug	June	July
hours	189	69	69	0	189	0	69	189	0	189	69	0
'cattiness'	788	620	376	341	274	203	201	197	197	149	142	135

	harvest year 2002								
extract	9	5	7	3	4	2	6	8	1
month	July	June	July	May	June	May	June	July	May
hours	189	69	0	189	0	69	189	69	0
'cattiness'	204	191	155	128	121	101	101	83	61

Table 3.2.xi. The Extracts Ordered from Highest Concentration of Thiol Level as Determined by GC FPD to the Lowest in the Harvest and Incubation Trials Conducted in 2001 & 2002.

Although there is some correlation between the cattiness as assessed by the aroma panel and the level of 4-methoxy-2-methyl-2-butanethiol there are some anomalies. Sample 7 from the 2001 season is ranked number 6 in the degree of cattiness by the aroma panel yet has the lowest level of the thiol. Conversely sample number 3 in the same year is ranked the lowest in it's degree of cattiness by the assessment panel yet has the 5th highest concentration of thiol out of the 12 samples. When the extracts were ranked from highest to lowest concentration of other individual components α-thujene was the only volatile whose level decreased in a pattern similar to the order shown in table 3.2.x.

3.2.4. Commercial-scale Incubation of Blackcurrant Buds for Improved Extract

The instigation of incubations on a commercial scale was undertaken by industry in the harvest year 2003. Large-scale extractions were completed for 8 batches and the results are recorded in table 3.2.xii.

sample	variety	treatment	thiol $mgkg^{-1}$	%oil yield
285	White Bud	incubated	31.4±0.3	3.56
286	White Bud	non-incubated	23.7±0.1	3.4
287	White Bud	incubated	41±4	3.78
288	White Bud	non-incubated	29±3	3.8
290	HTC	incubated	77±4	3.49
289	HTC	non-incubated	64.0±0.7	4.1
292	HTC	incubated	53.49±0.04	2.53
291	HTC	non-incubated	44.7±0.6	3.18

Table 3.2.xii. Thiol ($mgkg^{-1}$) and %Yield for Commercial Concretes from Incubated and Non-incubated Buds in 2003.

The levels of 4-methoxy-2-methyl-2-butanethiol increased by 29 % ± 5 (mean ± SE) whilst the oil yield decreased by 8 % ± 6. The oil yields obtained after incubation appeared to be less than non-incubated which was not apparent in pilot-scale experiments. However the increase in thiols confirmed the effectiveness in the release of this important component by post-harvest storage. The percentage compositions of individual component in the extracts from White Bud buds and HTC buds are presented in tables 3.2.xiii and 3.2.xvi respectively.

	%components in extract ($x10^{-3}$)			
	not incubated	incubated	not incubated	incubated
	286	285	288	287
α-thujene	103±9	103±2	64±2	88±3
α-pinene	389±12	336±8	414±22	461±19
sabinene	2782±76	3020±54	2925±85	1869±117
mycrene	361±9	338±0.006	365±13	422±14
	180±4	156±18	201±8	190±5
δ-3-carene	2971±71	2760±42	3533±125	3821±100
β-phellandrene & limonene	2020±43	1744±24	2421±89	2218±56
	397±9	362±8	477±13	428±12
	243±6	220±5	284±10	246±7
terpinolene	1631±30	1484±22	1874±54	1557±43
	129±2	139±5	132±2	187±4
β-caryophyllene	1636±12	1546±13	1472±14	1471±12
α-humulene	450±5	407±5	416±4	409±1
germacrene D	626±10	554±6	606±12	547±1
bicyclogermacrene	248±4	394±4	302±10	313±7
caryophyllene oxide	194±2	235±19	244,2	315±8
	156±2	138±4	122±1	160±1
polyanthic acid	3618±43	3464±26	3708±99	3769±38
hardwickic acid	31325±582	32172±31	26788±225	24457±243

Table 3.2.xiii. % Components in White Bud Buds Produced on a Commercial Scale

The relative yields of monoterpenes from non-incubated and non-incubated White Bud buds show inconsistent results. Extracts from incubated sample 287 are higher in most lower molecular weight monoterpenes than those from non-incubated paired sample, 286. This increase in monoterpenes is not evident in 285 (incubated) relative to 284 (not incubated). In both White Bud incubated / not incubated pairs the co-eluting α-phellandrene and limonene decrease as a result of incubation and this holds true for terpinolene and germacrene D. Not surprisingly caryophyllene oxide levels increase as a result of incubation. The results for the monoterpenes in the HTC clones are more consistent, with incubation increasing the levels for most major terpenes of this class. The results for the sesequiterpenes are less definitive though incubation has clearly increased the levels of germacrene D, caryophyllene oxide and hardwickic acid.

	%component in extract ($x10^{-3}$)			
	not incubated	incubated	not incubated	incubated
	289	290	291	292
α-thujene	74±1	105±1	104±9	150±3
α-pinene	221±12	212±6	301±23	494±1
sabinene	4254±211	5342±8	4526±213	5859±24
	168±4	171±2	223±20	329±2
mycrene	356±13	397±3	419±21	533±5
	91±5	75±1	130±6	168±5
δ-3-carene	2263±84	2369±14	2823±132	3746±30
	79±5	94±1	102±3	121±4
β-phellandrene & limonene	894±27	611±7	1343±64	1708±21
	375±9	419±4	381±17	411±9
	225±3	250±3	229±10	238±6
terpinolene	1224±13	1250±10	1505±65	1570±40
	159±1	266±6	202±3	282±5
β-caryophyllene	1127±12	1088±8	1650±37	1797±11
α-humulene	185±1	112±1	502±8	600±3
germacrene D	185±3	199±1	637±52	704±8
bicyclogermacrene	1394±20	1953±22	431±10	213±7
caryophyllene oxide	451±1	633±22	295±1	224±5
	83±1	99±8	161±1	201±5
polyanthic	3712±401	4369±65	3276±66	3125±6
hardwickic	28157±436	25242±509	25860±345	23213±33

Table 3.2.xvi. % Components in HTC Buds Produced on a Commercial Scale

Section 3.3. SYNTHESIS OF THIOLS

3.3.1. Synthesis of 4-Methoxy-2-methyl-2-butanethiol

The synthesis of 4-methoxy-2-methyl-2-butanethiol was undertaken to provide a standard to establish accurate standard curves to be used in the quantification of the thiol in blackcurrant buds and for reference in determining threshold levels in aromatic profiling.

Stage 1.

Chloromethyl ether was reacted with isobutene in a hermetically sealed reaction vessel. The solution was distilled under light vacuum (50 mmHg) to produce four fractions.

Fraction 1 - 32°C to 50°C – weight – 51.6 g

Fraction 2 - 50°C to 65°C – weight – 27.0 g

Fraction 3a - < 65°C – weight – 10.3 g

Fraction 3b - < 65°C – weight – 15.1 g

Fraction 2 contained the target product, 2-chloro-4-methoxy-2-methylbutane representing a recovery of 10.5 %. The yield reported by Riguard *et al.*, (1986) was 41 %. The chromatogram is presented in figure 3.3.i. The mass spectrum of 2-chloro-4-methoxy-2-methylbutane is shown in figure 3.3.ii. The conditions under which the chromatogram and mass spectrum were obtained are detailed in materials and methods, section 2.3.1. The identification was based on published spectra from NIST (Anon, 2001) and from a comprehensive 'in house' data base accumulated at the University of Tasmania through the Central Science Laboratory (pers. comm. N. W. Davies).

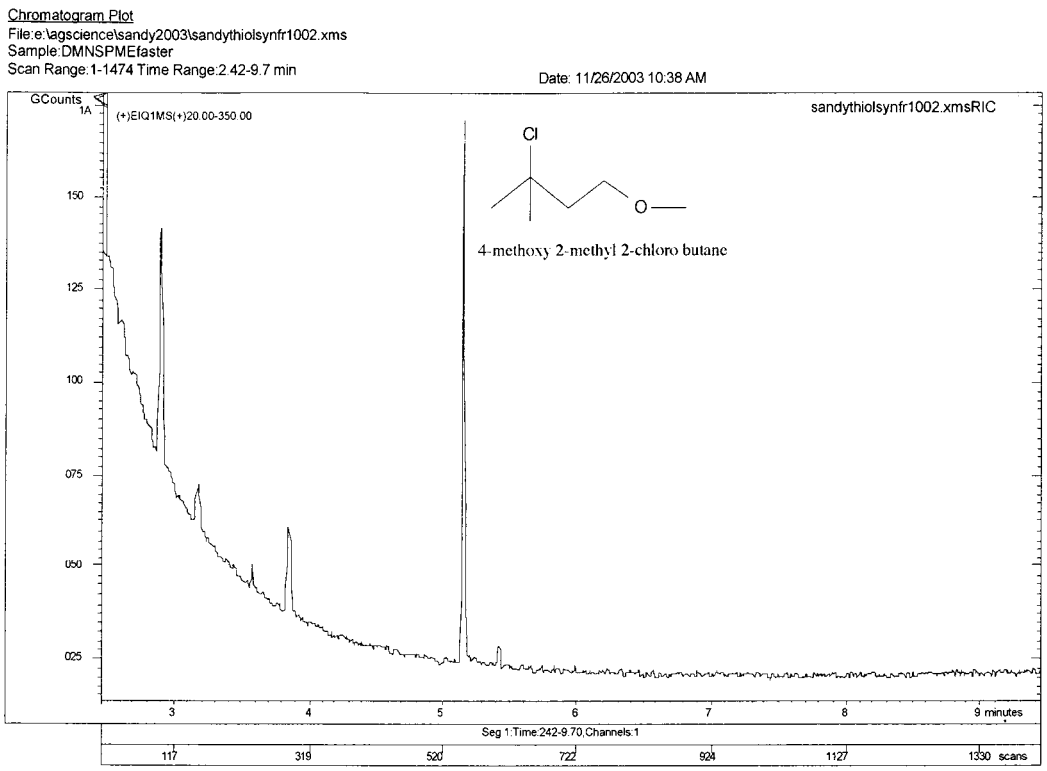


Figure 3.3.i. The GC MSD Chromatogram of Fraction 2 Containing Synthetic Thiol.

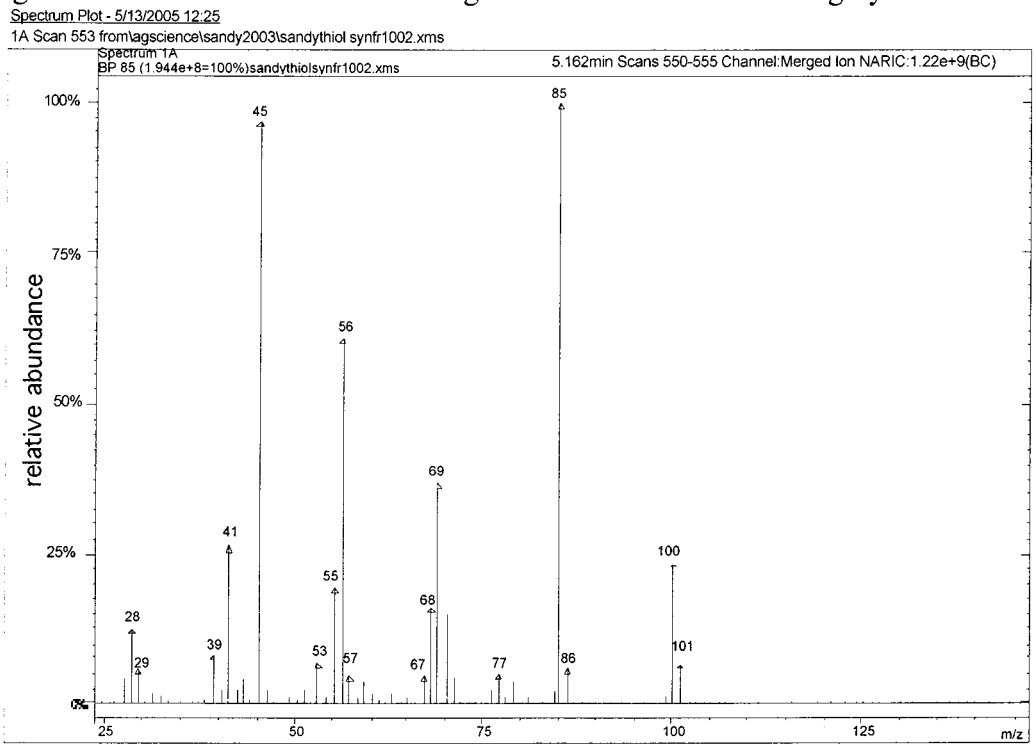


Figure 3.3.ii. The Mass Spectrum of 2-Chloro-4-methoxy-2-methylbutane from the Peak Eluting at 5.16 Minutes in Figure 3.3.ii.

The fragmentation of the 2-chloro-4-methoxy-2-methylbutane ($C_6H_{13}ClO$, M.W.=136) within the mass spectrometer was complete and the parent ion was not evident in figure 3.3.ii. Figure 3.3.iii. shows the chromatogram plots of the total ion current in

the top panel whilst the corresponding chromatograms of ions 121 and 135 are displayed in the two lower panels. Ion 135 is the parent ion having lost one proton and is clearly more evident at this higher resolution than in figure 3.3.ii. Ion 121 is produced by the loss of a methyl group. The presence of both ions confirm the successful synthesis of 2-chloro-4-methoxybutane.

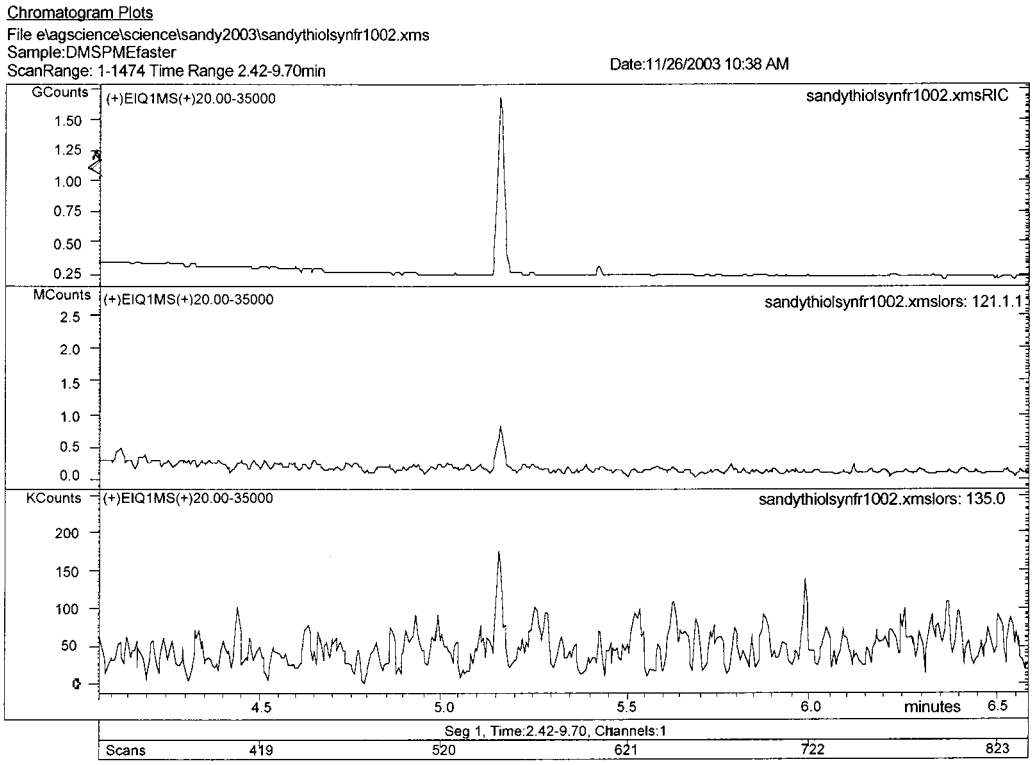


Figure 3.3.iii. The Total Ion Current and Ion Currents of Ions 121 and 135 Produced from the Analyses of 2-Chloro-4-methoxy-2-methylbutane using GC MSD.

Stage 2.

The second stage of the synthesis of 4-methoxy-2-methyl-2-butanethiol was unsuccessful. Although the synthesis was repeated several times the target product was only detected once and at very low levels. The yield obtained by Riguard et al., (1982) was 16.3 % for the second step of the synthesis with and overall yield of 6.6 %. An alternative method of synthesis was designed to achieve increased quantities.

3.3.2. A Novel Synthesis of 4-Methoxy-2-Methyl-2-Butanethiol.

When methyl-3-methoxypropionate is reacted with a Grignard reagent the methylated ester of the propionic acid is methylated at C1 and the intermediate ketone is then reduced to an alcohol. This process, as described in section 2.3.2, yielded 4-methoxy-2-methyl-2-butanol. The %yield was 58.6 with the purity of 94 % as assessed by GC. The chromatogram obtained by GC MSD is shown in figure 3.3.iv.

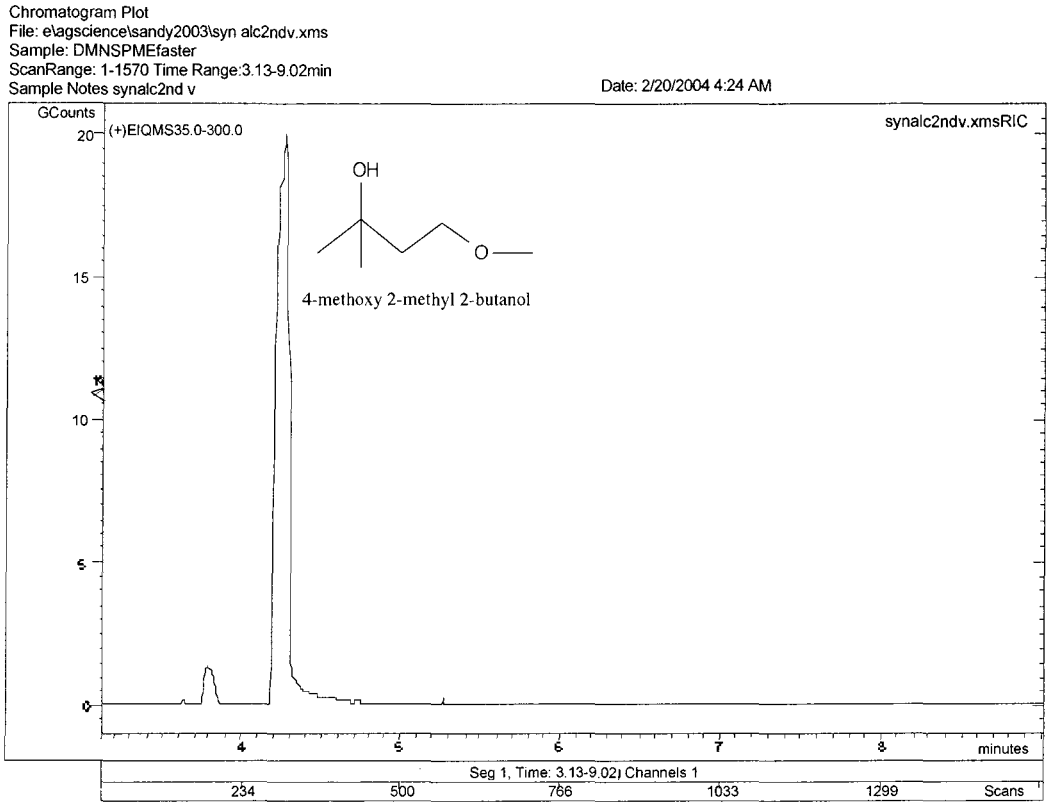


Figure 3.3.iv. The Chromatogram of 4-Methoxy-2-methyl-2-butanol by GC MSD.

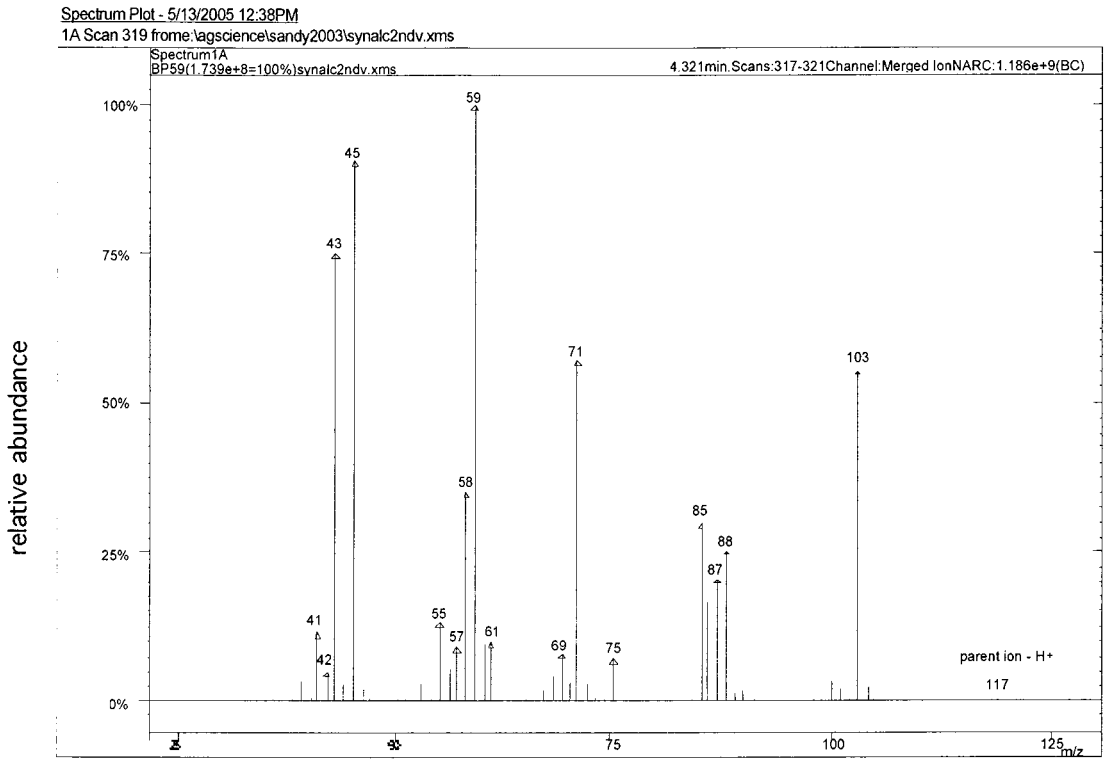


Figure 3.3.v. The Mass Spectrum of 4-Methoxy-2-methyl-2-butanol.

Stage 2.

The 4-methoxy-2-methyl-2-butanol was thiolated using Lawesson's reagent in toluene. The fractions collected were

Fraction 1 - 25 °C to 50 °C – weight – 6.14 g

Fraction 2 - 50 °C to 66 °C – weight – 8.8 g

Fraction 3 - < 65 °C – weight – 1.66 g

Fraction 4 - < 55 °C – weight – 0.91 g

Unfortunately the yields could not be calculated, as the thiol was not separated from the toluene, however, the response for the thiol relative to toluene as determined by GC was good with very few co-contaminants evident. Figure 3.3.vi shows the chromatogram of the synthesized thiol. The mass spectrum is displayed in figure 3.3.vii.

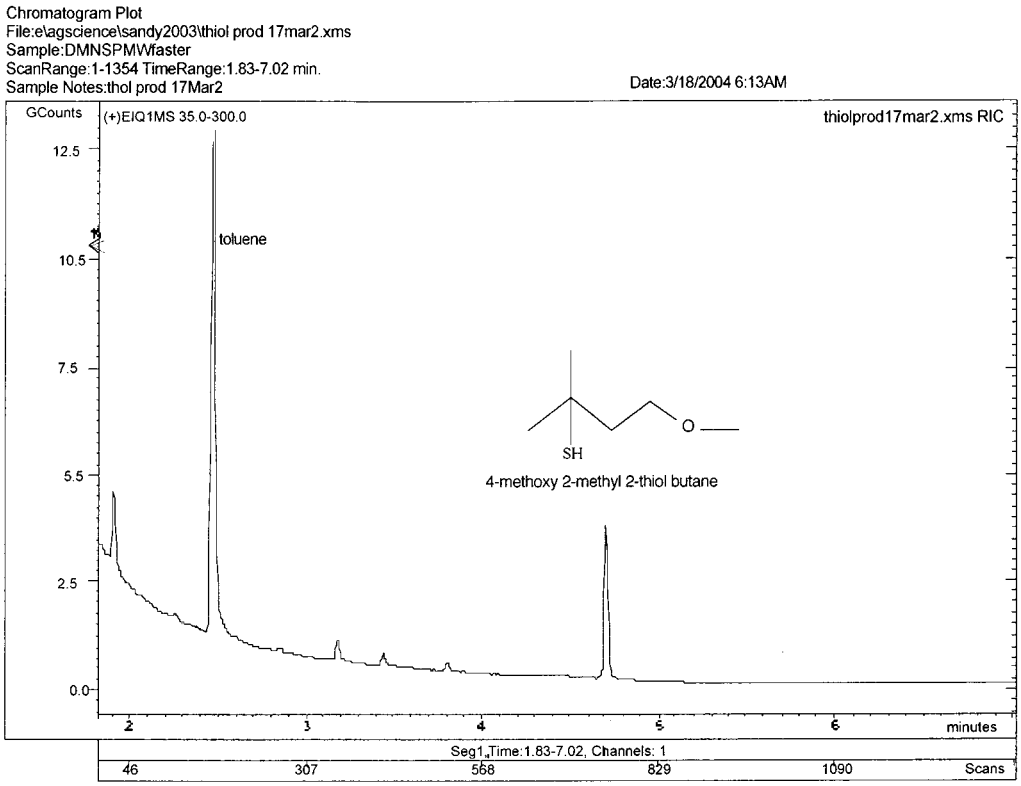


Figure 3.3.vi. The Chromatogram of Synthesised 4-Methoxy-2-methyl-2-butanethiol.

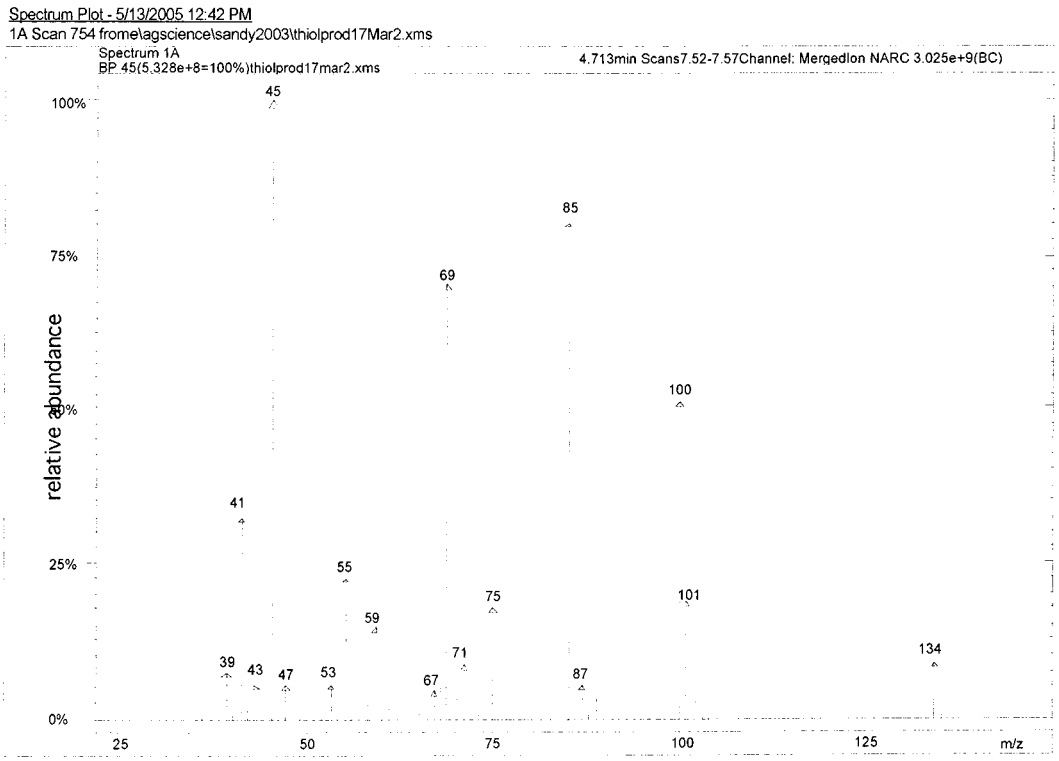
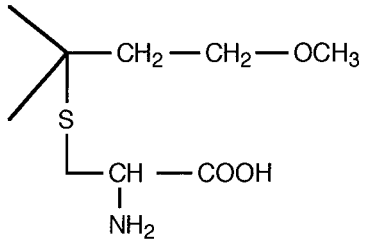


Figure 3.3.vii. The Mass Spectrum of the Synthetic Form of the Thiol Endogenous to Blackcurrants.

Section 3.4. SYNTHESIS OF THE CYSTEINE-THIOL CONJUGATE AND MONITORING OF THE THIOL PRECURSOR IN BLACKCURRANT BUDS AND EXTRACTS.

Research conducted in the viticulture industry has shown that some volatile pungent thiols are released from non-volatile cysteine conjugates. The similarity between the thiols detected in fermented wine must (un-fermented grape juice) of Sauvignon blanc and the thiols detected in blackcurrants leads to conjecture that the precursor to the thiol endogenous to blackcurrants is a cysteine conjugate with the structure as follows;-



As the thiol is present at low concentrations in blackcurrant it is probable that the level of any potential precursor is also low. To facilitate the establishment of the characteristic chromatographic properties and fragmentation patterns by mass spectrometry the synthesis of a 4-methoxy-2-butanethiol / cysteine conjugate was undertaken.

3.4.1.i. Synthesis of 4-methoxy-2-methyl-2-butanethiol / cysteine conjugate

The synthesis of the 4-methoxy-2-methyl-2-butanethiol cysteine conjugate was undertaken by reacting L-cysteine with the chlorinated methylmethoxybutane in a caustic solution (Materials & Methods 2.4.1. method 2).

Analyses by HPLC MS/MS showed the successful synthesis of the conjugate with the chromatogram of the total ion trace (panel A) and the trace for ion 222 (panel B) extracted from the TIC presented in figure 3.4.i. The mass spectra are recorded in figure 3.4.ii. and 3.4.iii.

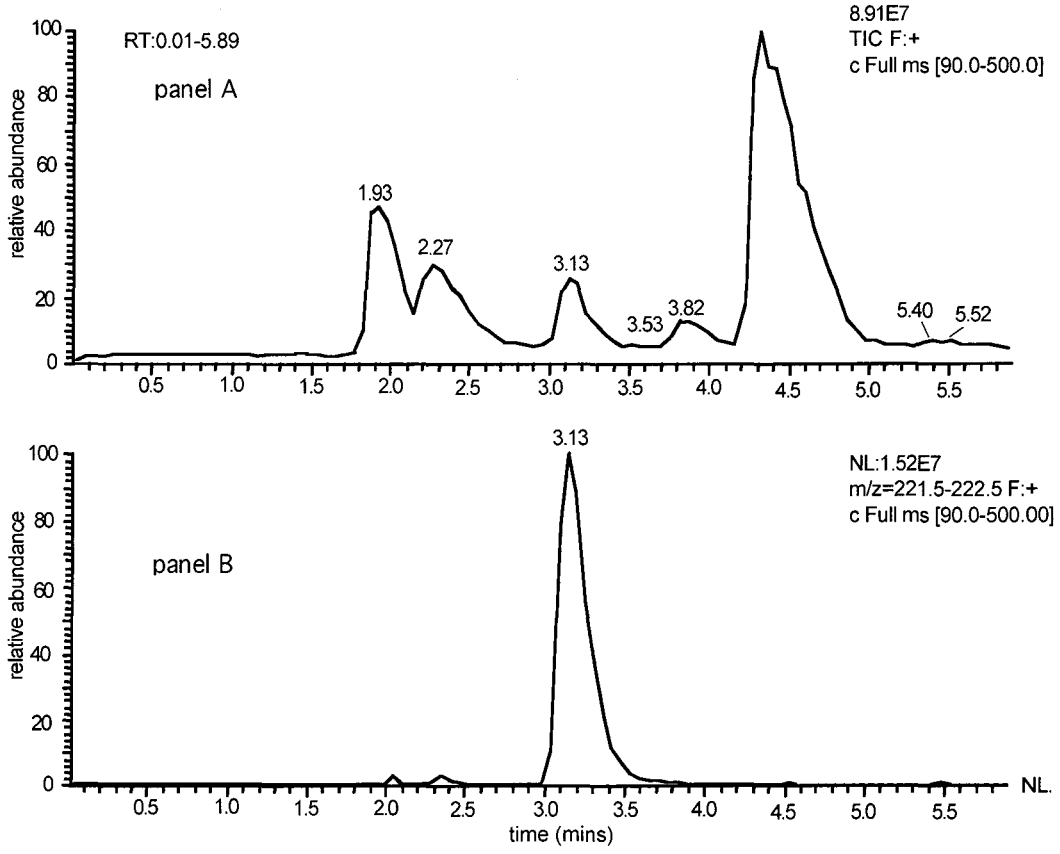


Figure 3.4.i. Panel A - Chromatogram of the Total Ion Trace of the Synthetic Cysteine – 4-methoxy-2-methyl-2-butanethiol as analysed by LC MSMS. Panel B - Chromatogram for Ions Extracted with a Mass to Charge Ratio of 221.5 to 222.5.

Panel A shows the chromatogram of all the ions formed in the nebulising chamber of the mass spectrometer as they eluted from the HPLC. The molecular weight of the protonated cysteine conjugate was 222. When ions with a mass to charge ratio (m/z) of 222 were extracted (panel B) the peak evident in panel A and eluting at 3.13 minutes was still present. The mass spectrum of the peak with a retention time of 3.13 minutes was consistent with the m/z of the cysteine-thiol conjugate and this is

displayed in figure 3.4.ii. To further validate the successful synthesis and identification of the precursor the ion from the parent molecule was further fragmented using a collision energy of 18 %. This produced the cysteine fragment with m/z of 122 displayed in figure 3.4.iii. In addition the ion fragment with an m/z of 101, consistent with a 4-methoxy-2-methylbutane ion, is also present.

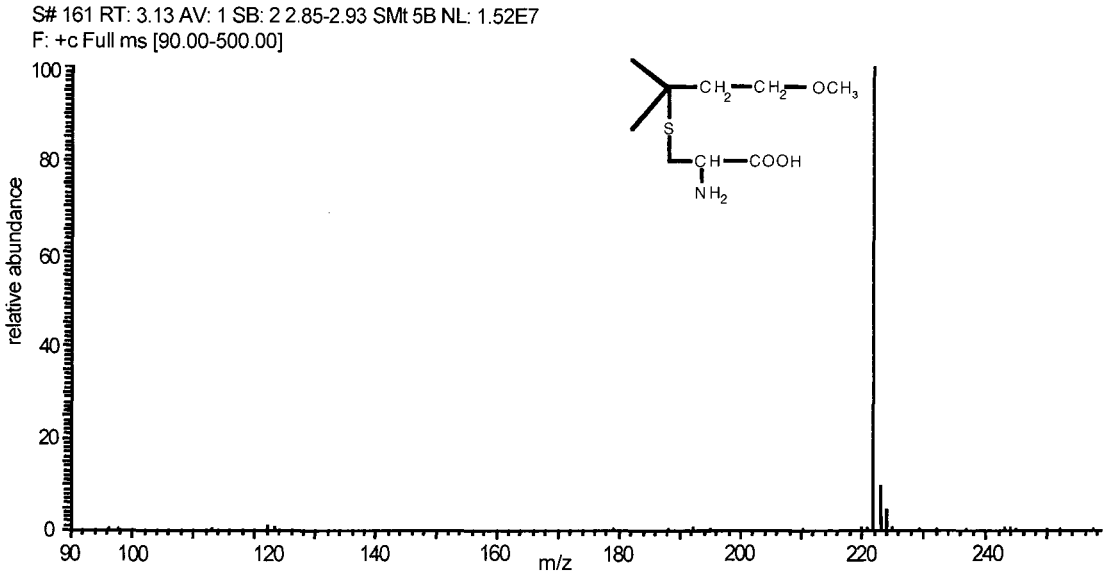


Figure 3.4.ii. Mass Spectrum of the Cysteine-thiol Conjugate

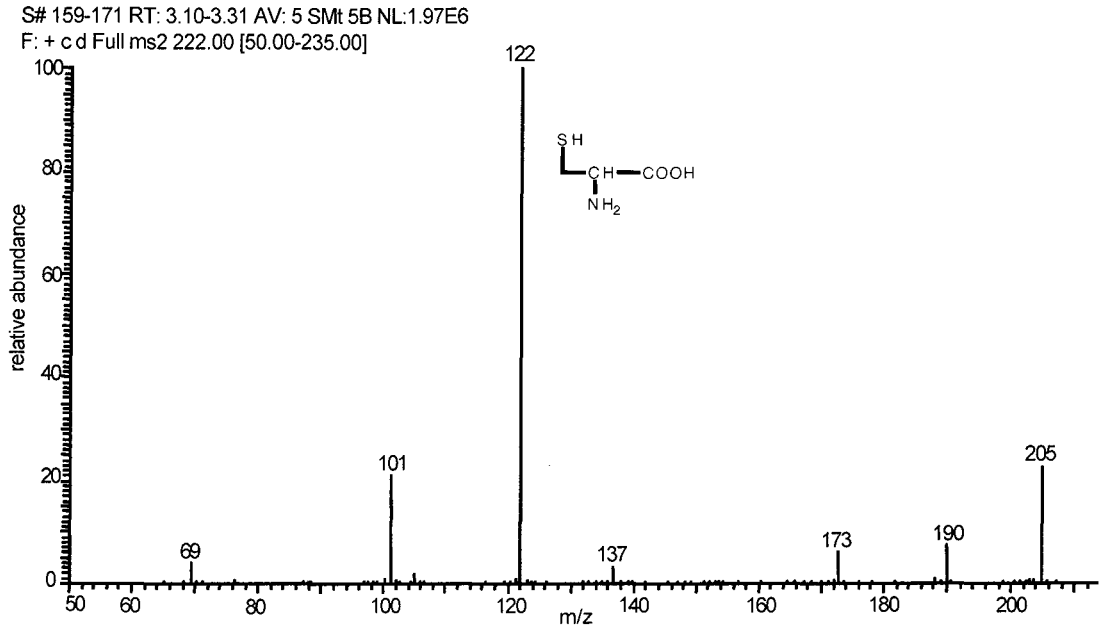


Figure 3.4.iii. Daughter ions of Mass Spectrum of the Cysteine-thiol Conjugate

3.4.1 ii. Clean-up of the Synthetic 4-Methoxy-2-methy-2-butanethiol Cysteine Conjugate Synthesised from the 4-Methoxy-2-chloro-2-methylbutane

The processes undertaken to purify the cysteine conjugate were unsuccessfull. In method 1, Materials & Methods section 2.4.1.ii, the solution in which the conjugate was applied to the C_{18} column was neutralised to maximise the interaction between the non-polar moieties of the target molecules with the C_{18} functionalised silica. The 1 % ethanol in water was too polar and a wash of 100 % methanol was required to elute the conjugate from the column. The recovery was poor indicating that the conjugate may have been degraded or may have precipitated out and been removed in the filtration step.

In method 2, Materials & Methods, section 2.4.1.ii, the conjugate was solvated in an acidic solution so that the amino acid was present as the cation $^{+}H_3NCHRCOOH$, enabling the aqueous solution to be washed with dichloromethane. Half of the solution was applied to a cation exchange column, washed with distilled water and eluted in ammonia solution. Recovery was poor. The remaining half was applied to a C_{18} column and again washed with 1 % aqueous ethanol. Applying the conjugate in acidic solution did improve recovery confirming that the cation was more susceptible to removal from the column by polar solvents. However the fragmentation of the parent cysteine-thiol conjugate in the HPLC mass spectrometer produced a ratio of daughter ions differing from that of the original synthetic product. Because of the susceptibility of the conjugate to degradation and rearrangement and the confidence in the correct identification of the chemical established by HPLC MS/MS, the experiments to establish a clean-up method were not continued.

3.4.1.iii. Synthesis of the Cysteine-thiol Conjugate using 4-Methoxy-2-methyl-2-butanol as the Starting Product.

4-Methoxy-2-methyl-2-butanol was reacted with l-cysteine in trifluoroacetic acid. The synthesis resulted in a more concentrated product. Any future purification experiments will use this synthetic process as the starting point.

3.4.2. Development of an Extraction and HPLC MS/MS Method for the Detection of the Cysteine-thiol Conjugate.

3.4.2.i. Flash Chromatography for the Isolation of Endogenous Cysteine-thiol Precursor from Blackcurrant Buds with 1 % Ethanol in Water as the Mobile Phase.

The experiment described in Materials & Methods, section 2.4.2.i did not result in the detection of the cysteine-thiol conjugate in blackcurrant buds. The experiments detailed in 3.4.1.ii. determined that the synthetic analogue was only slightly soluble in 1 % ethanol unless present in ionic form, with signs of lability and a propensity for rearrangement all contributing to poor recoveries. Many other investigative experiments were conducted using a variety of extraction techniques with the inclusion of the dry down of the extracting solvents by freeze drying, antioxidants and the chelation of the target compound on specialized columns. Despite the failure to detect the conjugate confidence remained that the cysteine based precursor was present in blackcurrants due to the similar structure of the thiol to those detected in wine. Sulphiting processes are included in the production of wine and research was undertaken to ascertain a sulphiting procedure that would be suitable to include in the extraction of blackcurrant buds.

3.4.2.ii. The Inclusion of Sulphiting in the Extraction of Thiol-cysteine Conjugate from Blackcurrant Buds.

Within the wine industry grapes are preserved with antioxidants and acidification agents to ensure the stability of the juice. In addition, maintaining a pH of 3 is considered critical for stability through the processing stages. Sodium metabisulphite, ascorbic acid and tartaric acid were applied in the extraction process of blackcurrant buds to preserve the cysteine thiol-conjugate. Blackcurrant buds were extracted in additive solution containing these three chemicals and applied to columns containing octadecyl functionised silica and eluted with 1 % ethanol in distilled water. Synthesised cysteine conjugate was used to fortify 500 μL of 1 % ethanol in distilled water as a reference (sample A). An equivalent quantity was used to fortify dilute additive solution that was also applied to one of the C_{18} columns and eluted with 1 % ethanol in distilled water (sample B). Freshly cut blackcurrant buds were fortified

with synthetic conjugate and extracted in additive solution applied to a C_{18} column and eluted with 1 % ethanol in distilled water (sample C). Finally freshly cut blackcurrant buds that had not been fortified with synthesised conjugate were extracted in the same manner as sample C (sample D). Table 3.4.i lists the response values for the 4 samples analysed.

sample	description	response of cysteine (peak area)	response of cysteine conjugate (peak area)
A	10μL conjugate in 500μL 1% ethanol in water	127399555	2421743
B	10μL of conjugate in 9 mL H_2O + 1mL additive soln.-column	31596038	955000
C	10μL of conjugate in blackcurrant extract with additive soln.-column	0	169965
D	Blackcurrant in additive soln.-column	0	427201

Table 3.4.i. Response Values of Samples Fortified with the Conjugate 4-Methoxy-2-methyl-2-thiol Butane – Cysteine.

The results listed in table 3.4.i were un-expected. The detection of the conjugate in sample D indicated the successful identification of the proposed thiol precursor in blackcurrant buds. However sample C, which should contain endogenous thiol as well as the amount of thiol used to fortify the sample, had a lower result than the un-fortified sample.

Re-extraction of endogenous cysteine-thiol conjugate - Further experimentation was undertaken to confirm the positive identification of the cysteine-thiol conjugate in blackcurrant buds. Measures were taken to ensure that the buds to be extracted were not contaminated with the synthetic conjugate. Extractions were conducted using an increased concentration of additive solution and extraction conditions were implemented to ensure minimum potential for oxidation of the target chemical. Two samples were prepared. Unfortified blackcurrant extract and fortified extract. The

samples were analysed in conjunction with a reference sample of the synthetic cysteine conjugate in distilled water. The two columns used for each extraction were also washed with methanol. The methanol wash was also analysed to ascertain whether all of the conjugate had been eluted from the column. The results are presented in table 3.4.ii.

sample	description	response for cysteine conjugate (peak area)
1	blackcurrant extract (no synthetic conjugate)	4370717
2	methanol wash	0
3	blackcurrant extract + synthetic conjugate	5541542
4	methanol wash	0
5	standard synthetic conjugate	1874274

Table 3.4.ii. Response Recorded for Endogenous and Synthetic Cysteine-thiol Conjugate in Blackcurrant Buds as Analysed by LC MS/MS.

When the mass spectrometer accumulates ions with specific molecular weights, ions that have molecular weights not corresponding to the target m/z are expelled from the ion collection chamber. By increasing the oscillations of the ions within the ion chamber the mass spectrometer has the capacity to impel the further fragmentation of the original target ions. The energy required to bring about this controlled fragmentation is called the collision energy (CE). The target molecule in this case is the cysteine-thiol conjugate which has a molecular weight of 221 with a m/z ratio of 222 when ionised. The accumulation of molecules with this m/z is followed by the increase in collision energy to 18 % which results in the fragmentation of ion m/z 222 to the smaller ions with m/z 122 ($C_3H_7NO_2S$ MW - 121) and m/z 101 ($C_6H_{12}O$ MW – 100). Figures 3.4.iv and vi show the MS/MS chromatogram acquired from a blackcurrant extract that had not been fortified with the synthetic cysteine-thiol conjugate and the chromatogram from synthetic cysteine-thiol conjugate fortified into

blackcurrant extract respectively. The mass spectrometer scanned for ions between 100.5 to 101.5 and ions 121.5 to 122.5 produced by the fragmentation of the parent ion 222.00. The parent ion 222 corresponds to the protonated form of $C_9H_{19}NO_3S$ (MW of 221) with m/z 122 characteristic of the fragment, cysteine ($C_3H_7NO_2S$, MW - 121) and m/z 101 resulting from the methoxy-methylbutane moiety ($C_6H_{12}O$, MW – 100).

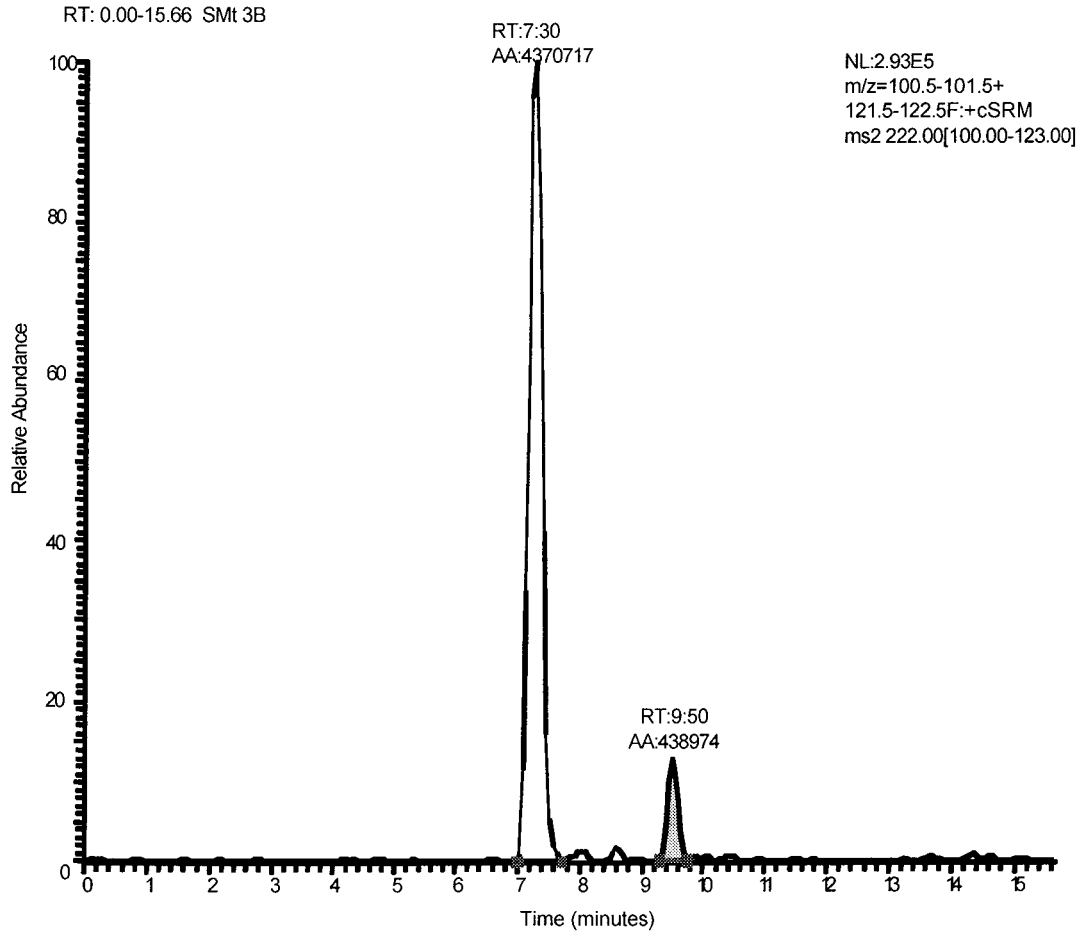


Figure 3.4.iv. The HPLC MS/MS Chromatogram of Cysteine Conjugate from Un-fortified Blackcurrant Extract.

Figure 3.4.iv shows the chomatogram of a blackcurrant extract that was not fortified with synthetic cysteine-thiol conjugate. The accumulation of ions with an m/z corresponding to the controlled fragmentation of the cysteine-thiol conjugate resulted in a strong peak with retention time 7.30 minutes. This correlated well with the chromatogram shown in figure 3.4.v, in which the synthetic form of the target

chemical had been used to fortify blackcurrant extract. The excellent correlation was convincing evidence of the presence of the precursor in blackcurrant.

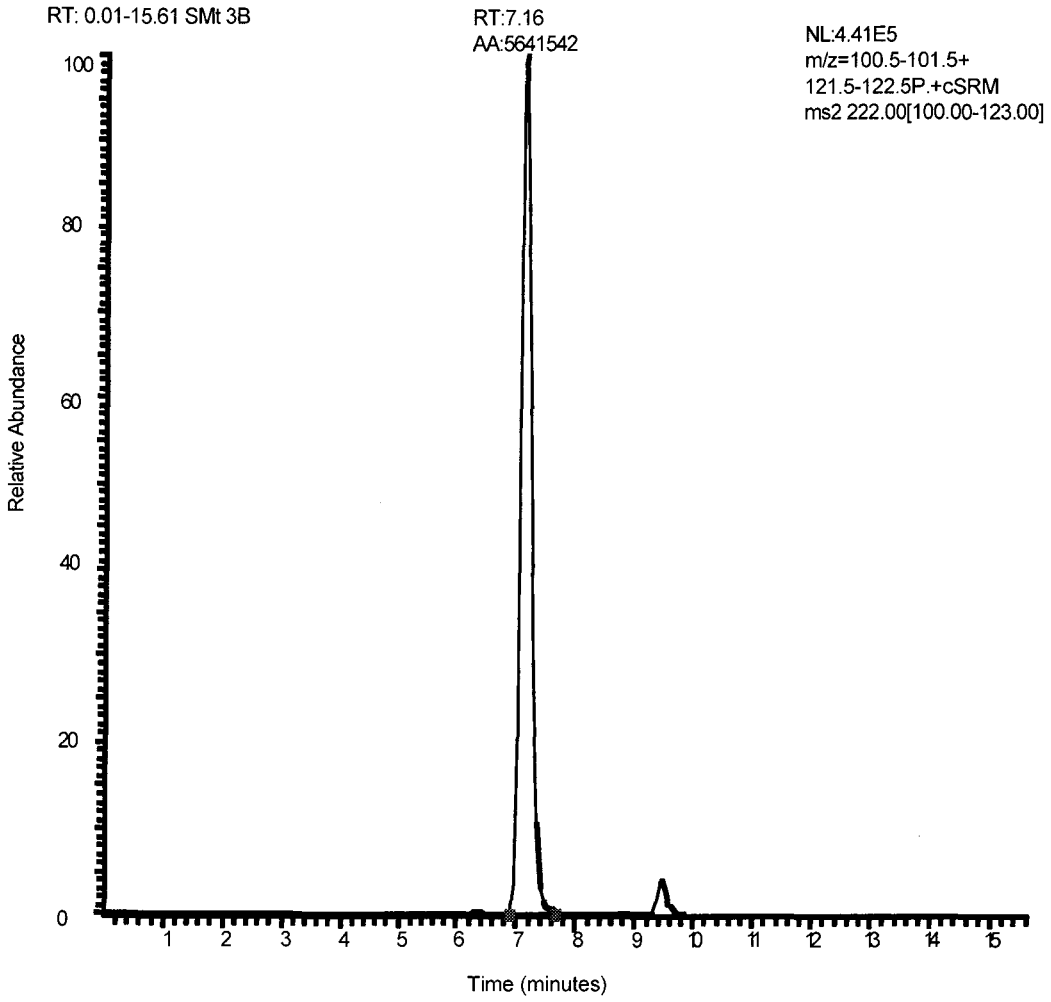


Figure 3.4.v. The MS/MS Chromatogram of Blackcurrant Extract Fortified with the Synthetic Cysteine – thiol Conjugate

The final figure (3.4.vi) in this series of chromatograms is of the synthetic cysteine-thiol conjugate in solvent analysed using the same HPLC MS/MS protocol but without the presence of blackcurrant extract. When the area of the peak eluting at 7.3 minutes in figure 3.4.iv of 43.7 x e^6 (endogenous conjugate) was subtracted from the area of the peak eluting at 7.2 minutes in figure 3.4.v of 56.4 x e^6, the response of the peak attributable to synthetic conjugate was 2.7 x e^6. The area of the synthetic standard in solvent (figure 3.4.vi) of 1.9 x e^6 correlated reasonable well with this expectation.

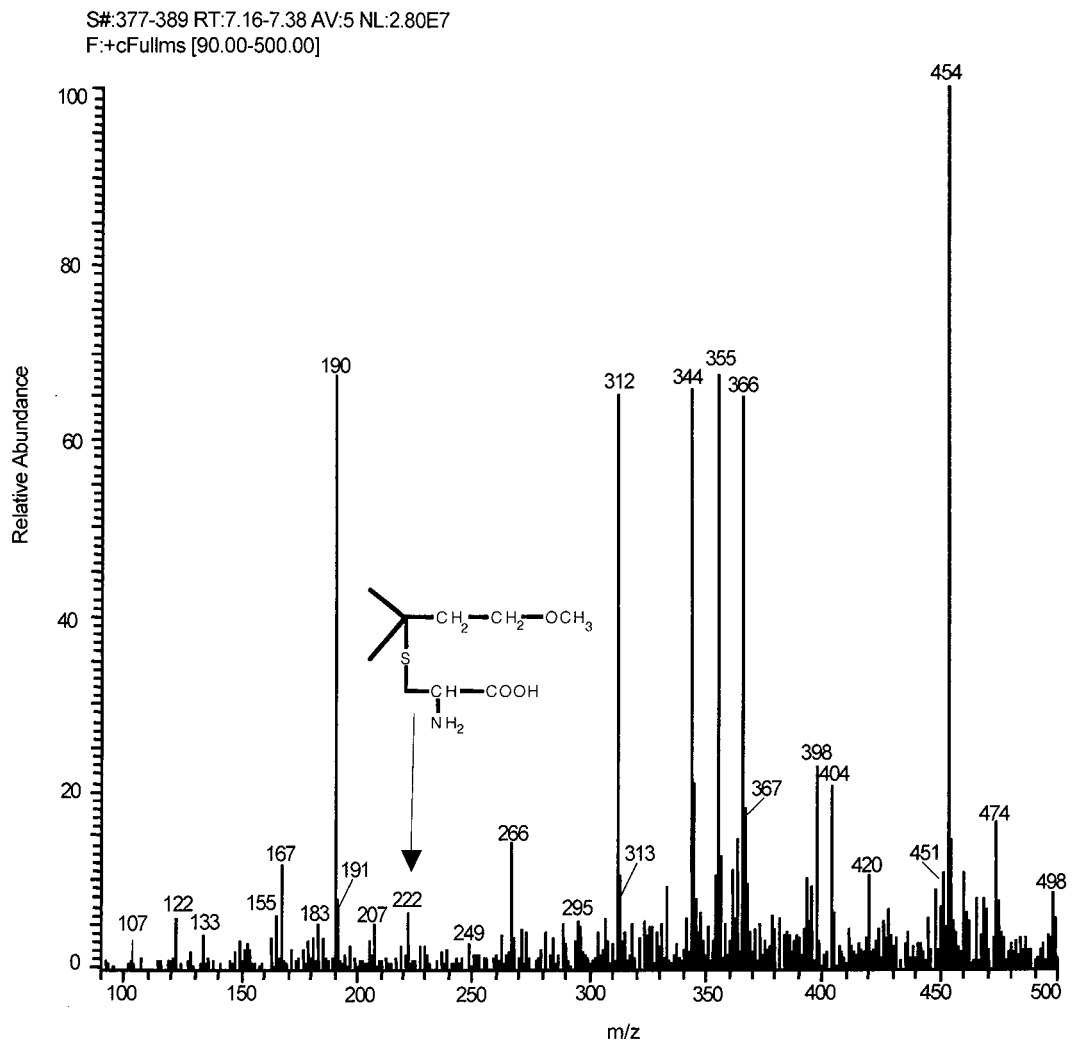


Figure 3.4.vii. The Chromatogram of the Full Scan of Blackcurrant Extract Showing Peak with Mass 222 Corresponding to the Cysteine Conjugate

Two mass spectra are presented in figure 3.4.viii. Panel A present the mass spectrum of the daughter ions fragmented from ion m/z 222 extracted from the chromatogram from blackcurrant extract that had not had synthetic cysteine-thiol conjugate added. It represents the mass spectrum of a chemical endogenous to blackcurrants. Panel B corresponds to the mass spectrum of ions extracted from the parent ion m/z 222 of the synthetic analogue. The correlation of the ions registered and the characteristic ratios common to both spectra in panels A and B provided convincing evidence that the target chemical is present in blackcurrant and is the likely precursor to 4-methoxy-2-methyl-2-butanethiol.

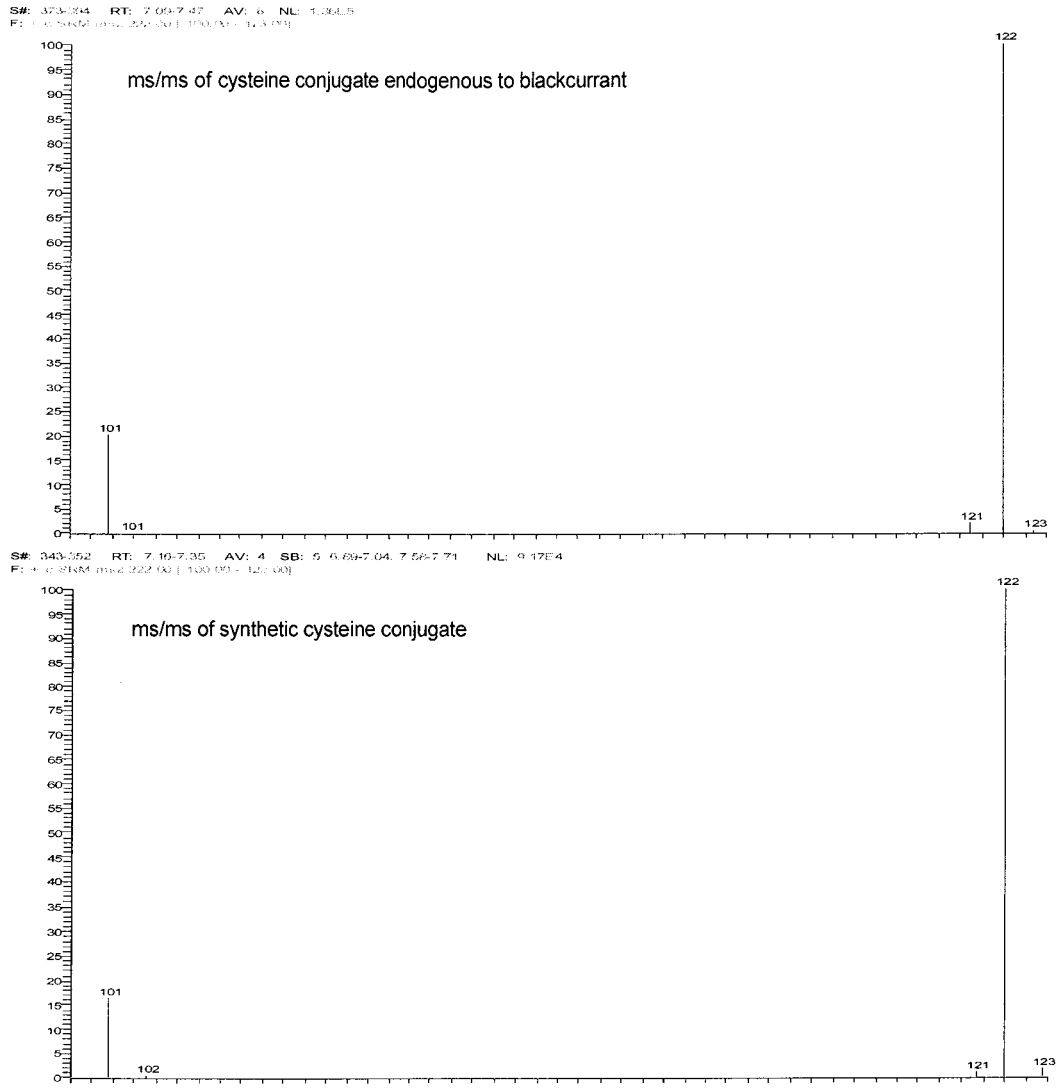


Figure 3.4.viii . Mass Spectra of the Daughter Ions from Parent m/z 220 Detected in Un-fortified Blackcurrant Extract (panel A)and from the Reference Synthetic Cysteine-thiol Conjugate (panel B).

3.4.2.iii. Repeatability of the Extraction and Quantification of the Thiol Conjugate in Blackcurrant Buds.

In order to determine if the extraction method developed for the thiol conjugate from blackcurrant buds was quantitative and repeatable the extraction process was conducted in triplicate. The samples prepared were also analysed by GC FPD to determine the levels of endogenous thiol. The results indicated that the process was

not quantitative. The values of the three replicates as determined by HPLC MS/MS are listed in table 3.4..iii along with the levels of endogenous thiols.

sample	thiol level (GC FPD)(mgkg^{-1})	cysteine-thiol conjugate response (peak area x10^6)
1	11.26	1.05
2	4.9	0.24
3	4.77	0.18

Table 3.4.iii. Level of Endogenous Thiol as Determined by GC FPD and the Response for Cysteine–thiol Conjugate in Fresh Blackcurrant Buds Analysed by LC MS/MS.

The results listed in table 3.4.iii indicated that the extraction method could not be used in a quantitative study. There did appear to be some anomaly in the results for the analyses of thiols by GC FPD and in fact the responses by HPLC MS/MS repeated, to some extent, the variation recorded across the GC FPD results. None the less the selectivity and specificity of the HPLC MS/MS potentially allowed for the omission of the chromatographic step in the extraction of the cysteine-thiol conjugate thereby reducing the potential for loss of analyte and increasing the repeatability of the analyses.

3.4. 2.iv. The Recovery and Repeatability of the Analysis to Quantify the Levels of Cysteine-thiol Conjugate When Flash Chromatography is Excluded.

The high degree of specificity and low detection levels achievable by HPLC MS/MS presented the potential to omit the clean-up of the blackcurrant extracts using flash chromatography. Table 3.4.iv list the results for the repeatability experiment for the extraction and direct analysis of the cysteine-thiol conjugate from blackcurrant buds.

sample	thiol level (GC FPD)(mgkg^{-1})	cysteine-thiol conjugate response (peak area x e^{6})
1	23.92	6.33
2	24.77	7.88
3	26.26	9.22

Table 3.4.iv. The Level of Endogenous Thiols and the Response of the Cysteine-thiol Conjugate Detected in Blackcurrant Buds by HPLC MS/MS

The inter-sample variability was still high at 30 %. In subsequent experiments the concentrations of the additive solution was increased. However the experiment detailed in this section has confirmed that the chromatographic step in the extraction process does not compromise the analytical methodology.

3.4.3. The Relative Concentration of the Cysteine-thiol Conjugate in White Bud and HTC Blackcurrant Buds.

The levels of endogenous thiols have been found to be significantly higher in the high thiol clones propagated at the University of Tasmania relative to the variety used in most commercial plantation, cv. White Bud. If the endogenous thiol level is high it may be proposed that the level of conjugate may also be disparate. The levels of thiol and cysteine-thiol conjugate in buds from HTC and White Bud were determined using GC FPD and HPLC MS/MS Materials & Methods 2.4.3) and the results are presented in table 3.4.2.i.

Sample	thiol concentration $mgkg^{-1}$ (DMB)	conjugate response (peak area x e^3) / bud weight
HTC 1	12.7	164
HTC 2	14.6	140
HTC 3	14.2	149
HTC 4	14.2	208
White Bud 1	4.4	36
White Bud 2	4.4	30
White Bud 3	4.1	43
White Bud 4	4.1	36

Table 3.4.3.i. Levels of Endogenous Thiol in Blackcurrant Buds Harvested from HTC and White Bud and the Response by HPLC MS/MS for the Endogenous Cysteine-thiol Conjugate.

The level of the endogenous 4-methoxy-2-methyl-2-butanethiol in HTC buds was 3.2 fold the level detected in White Bud. The corresponding level of the cysteine-thiol conjugate was 4.5 fold the level detected in White Bud. The direct relationship between high thiol levels and high cysteine-thiol conjugate levels provides further proof that the precursor has indeed been identified and presents the possibility to develop an assay to determine the potential for thiol production in blackcurrants.

3.4.4. Monitoring the Endogenous Thiol and Cysteine-thiol Conjugate in Blackcurrant Buds Prior to Bud Burst.

A time series trial to monitor the levels of the endogenous thiol and the change in concentration of the cysteine-thiol conjugate in blackcurrant buds was established at the trial site at the University of Tasmania farm. Buds were harvested on a weekly basis up until bud burst. Table 3.4.vi record the relative responses for the endogenous thiol and the cysteine-thiol conjugate levels form the 15th July to the 8th of October.

date	days	thiol $mgkg^{-1}$ mean ± SD (n=4)	conjugate response (peak area/bud weight x e^3) mean ± SD (n=4)
29-Jul	0	13.9±0.8	170±27
5-Aug	7	11.3±0.3	146±14
12-Aug	14	16.5±0.9	124±11
19-Aug	21	19.3±0.5	84±18
26-Aug	28	15.4±0.9	59±9
3-Sep	35	16.0±0.4	66±15
22-Sep	42	13.9±0.8	111±10
1-Oct	56	13.6±0.5	51±25
8-Oct	63	14.9±0.3	107±51

Table 3.4.vi. The Levels of Endogenous Thiol and Thiol-conjugate in High Thiol Buds During Bud Burst.

Figure 3.4.ix graphs the results listed in table 3.4.vi as a percentage of the amount of each component remaining relative the level detected at time zero

$$\%\text{component remaining} = \frac{\text{level detected at time X}}{\text{level detected at time zero}} \times 100$$

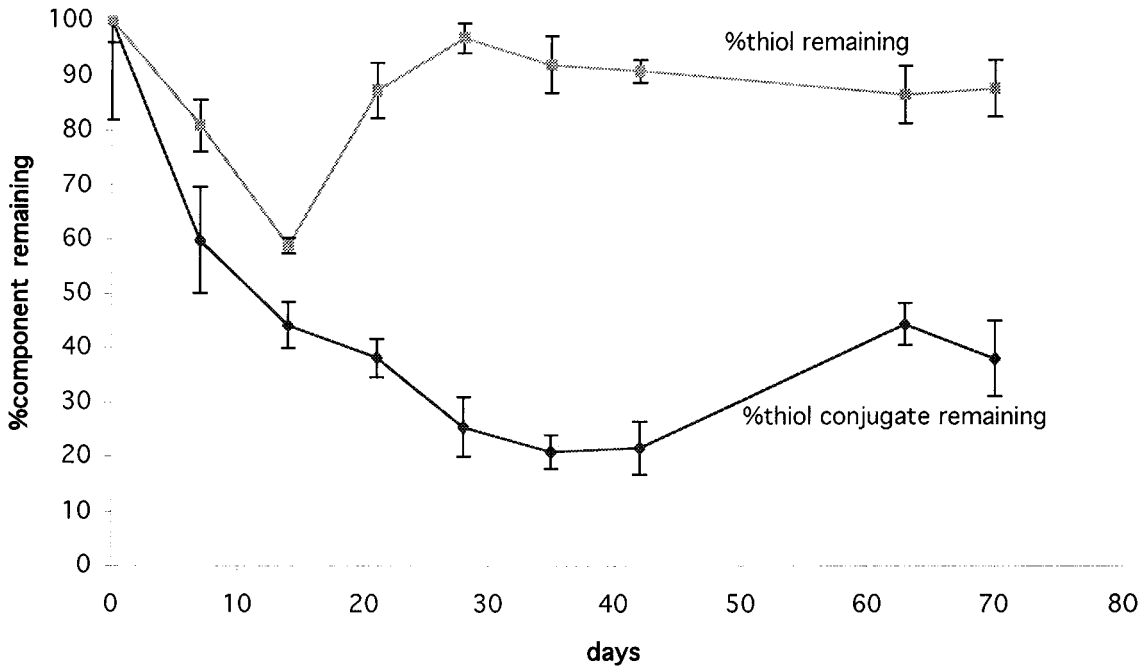


Figure 3.4.ix. The %Thiol and %Cysteine-thiol Conjugate Remaining in HTC Buds From Day Zero Over Time.

Although the results presented here were generated using analytical methods which required further development, the results indicate that there was an inverse relationship between 4-methoxy-2-methyl-2-butanethiol and the cysteine-thiol conjugate. This is not immediately apparent prior to the 21st of August (day 21) where the levels of both components decrease concurrently. The release of thiols subsequent to the 21st of August, and up until bud burst, was consistent with the increase in thiol levels reported in the dormancy and harvest experiments detailed in section 3.2.

DISCUSSION

Section 4.1. HARVESTING AND EXTRACTION TECHNOLOGY

4.1.1. Sieving Experiment.

The introduction of machine-harvesting has been instrumental in establishing a economically viable blackcurrant bud extract industry. The amount of co-harvested extraneous material, however, results in lower recoveries and, to some extent, a compromise in extract quality. The use of a sieving machine separated large amounts if the extraneous material including bark, cane shards and leaf from bud material. The 65 % recovery of sieved buds is similar to the 60 % of intact buds separated from machine-harvested buds by hand as reported in the study conducted by Menary (1990), suggesting that the process has emulated the separation of intact buds from other material by hand. The yields of blackcurrant extract from the intact buds extracted by Menary (1990) were much higher at 4.5 % compared to the yield of 2.9 % recorded in this study. The disparity may be due to a number of factors including less favorable seasonal conditions and the age of the plants. The 27 % yield of fines consisted of bud tips and fragments. When sieved buds were combined with the fines the total yield of 92 % collected from the original un-sieved machine-harvested product represented a good recovery of extractable material. The yield of concrete from the combined buds and fines was low at 2.4 % but still compared favorably to the 1.98 % of extract recovered from un-sieved machine harvested buds. To put this in context - a 1 kg sample of buds would return 20 g of blackcurrant extract. The 920 g of material sieved from the same sample would return 24 g of extract and use 8 % less solvent. In addition the removal of the absorbent fluffy winnow fraction may allow for the reduction in the amount of solvent required for efficient extraction.

Chemical profiling using GC indicated that the sieved buds had higher proportions of the thiol and most other quality component than all other fractions, including the un-sieved buds, though the relative compositions in the extract were similar. Of note is the higher proportion of caryophyllene oxide in the fines relative to the unsieved and sieved buds. This co-incides with lower concentrations of the endogenous thiol. The fines consist of bud fragments and tips from buds damaged by the mechanical harvester and it is likely that these fragments have increased exposure to oxidative

conditions. The effects of oxidation and its potential to alter the aroma profile of blackcurrant extracts are discussed more fully in the section dealing with post-harvest technology.

The essential oil industry needs to assess whether the added costs of passing blackcurrant harvests through the sieving machine is offset by the 20 % increase in extract yield along with the 10 % reduction in the amount of solvent required. This becomes more relevant as the price of extracting solvent increases. The installation of a sieving machine within the processing factory itself may make this alternative more attractive. The quality of the extracts produced is also a further consideration and future studies would benefit from complete aroma assessments.

4.1.2. Solvent and Maceration Methods

The experiments detailed in section 2.1.3 to 2.1.4 were undertaken to assess some of the procedures to be used routinely in subsequent experiments. The level of thiols in the buds available for this experiment were below detection limit and as this chemical is detected only by GC FPD the response recorded for 4-methoxy-2-methyl-2-butanethiol would not contribute to the yields calculated using the GC FID method described in section 2.1.4. So how did the methods of assessment using GC FID to analyse small laboratory scale experiments relate to the properties of the actual extracts produced? Indeed when the concentration of components detected by GC FID were calculated by relating the responses of all the peaks to that of an internal standard of known concentration, assuming a 1:1 response ratio, only 37 % the weight of the extract analysed was accounted for. The 63 % not detected by GC FID were non-volatile and therefore less likely to contribute to the aroma profile of the extract or were below detection limits. The main objectives of section 2.1 were to look at the comparative yield of quality components within the volatiles of blackcurrant extracts. In this respect GC FID provides a quantitative assessment, particularly when the yields of volatiles are reported relative to the bud weight and not as a proportion of the extract actually produced. This is not to understate the importance of the non-volatile components of the final extract. The viscosity and consistency of the extract are fundamental to aroma delivery and market acceptability.

The composition of the solvent had a marked effect with the yields declining as the proportion of the non-polar solvent, hexane, was added to petroleum ether. Hexane at a proportion of 1 % in petroleum ether returned a 17 % increase over 5 % hexane and a 37 % increase compared to the yield from 100 % hexane.

The method of maceration had a significant effect on the yield of extract from blackcurrant buds. The use of a stomacher, which massaged the buds, yielded 2.3 fold the calculated quantity of extract than that recorded for buds ground under liquid nitrogen and 1.5 fold that yielded from the rolled buds.

The experiments undertaken were not designed to improve the quality of extracts but were to determine whether yields measured by GC FID of solvated extracts were representative of yields determined by weighing final extracts. Solvated extracts cannot be used to indicate overall yield of extracts that have had the extracting solvent removed, however, the yield of volatiles and acids relative to the weight of buds can be established. Repeatable results, with improved yields, may be obtained by extracting in 5 % hexane in petroleum ether and by increasing the degree of homogenation.

4.1.3. Effect of Homogenisation Under Solvent

The assessment of alternative methods of homogenization showed that masceration of buds whilst submerged in solvent is far more effective in extracting volatile components than pulverizing in liquid nitrogen prior to solvent addition. Yield of components such as hardwickic acid and 20-oxo-hardwickic acid increased by three fold. It is recommended that all experiments investigating yield and composition of blackcurrant extracts undertake mascerations with buds immersed in solvent and using an omnimix or ultra-turrex.

4.1.4. Preliminary Experiments to Include the Steeping of Buds in Ethanol.

Unfortuately in this experiment any comparisons drawn between the extraction methods most be qualified as the method of homogenation used for the standard commercial method, using non-polar solvents alone, differed from the method of homogenation used for the ethanol based extractions. However the experiments

presented in section 3.1.10.iv (page 97) investigated the difference in extract yield and yield of volatiles from rolled buds and rolled buds that had also been subject to ultra-turrexing. The alterations to the solvents used were found to have a much greater effect on the yield of the extracts than did the change in the masceration methods. Hence, despite the differing masceration methods, the dramatic differences observed in aroma profile of extracts from buds steeped in ethanol compared to standard extraction methods, does warrants some attention. The aroma profile of extracts using the ethanol based method was very different with high impact components such as α-pinene, present at levels 10 times those recorded for the standard extraction method. The auto-oxidation of the monoterpene fraction in blackcurrant bud extracts has been demonstrated (Latrasse and Demaizieres, 1971) and the dramatic increases in many of the monoterpenes in this experiment indicate that the processes which instigate the depletion of such components may well be suppressed when ethanol is used as the preliminary extracting solvent. Yields of β-mycrene, δ-3-carene and β-phellandrene, three of the components reported by Latrasse to be susceptible to oxidation were all higher in the ethanol based extraction process. Indeed the levels of the co-eluting β-phellandrene and limonene were 45 % higher. Limonene has been found to be destroyed by enzymatic action during extraction and in work conducted at the University of Bristol this monoterpene was found to decrease slightly in blackcurrant bud extracts on storage (Williams, 1972). Although Williams did not improve the yield of limonene by using methanol as the preliminary extraction solvent the yield was higher when extractions were performed under a nitrogen atmosphere. The higher yield obtained in this study when ethanol was used provides a strong indication that enzymatic degradation is indeed retarded by the addition of low molecular weight alcohols. The yield of volatiles was higher in the ethanol extraction method detailed in section 3.1.5, though less acids were co-extracted. The amounts of components not detected by GC FID, and hence non-volatile, were slightly higher in the standard extraction protocol using only 5 % hexane in petroleum ether. The inclusion of ethylacetate in the extracting solvent was not effective yielding higher proportions of non-volatile components.

Experiments detailed in section 3.1.5, then, indicated that the inclusion of an initial step where buds are submerged in ethanol prior to maceration has potential, warranting further experimentation.

4.1.5. Effect of Filtering Extracting Solvent from Buds Prior to Partitioning into Non-polar Solvents

In standard operations the solvent is drained from the extraction drum, leaving behind the buds. With the adoption of the extraction method whereby ethanol is used as the preliminary extraction solvent, and where the subsequent polar extract is partitioned against a non-polar solvent, it would be a more expedient process to instigate the partitioning after the solvent was removed from the extraction drum. The effect on the final product of this sequence of processes is pertinent. The yield of concrete is increased by 13 % when buds are removed from the solvent prior to partitioning. Much of this may be attributed to an18 % increase of non-volatile components that are not amenable to GC, though the yield of acids decreased. The extracts produced from this process were found to be more catty with a strong blackcurrant background. The increased cattiness coincided with higher levels of 4-methoxy-2-methyl-2-butanethiol that was 65 % higher that the level detected in extracts produced whilst buds were present through the partition (table 3.1.viii, page 87). These lacked cattiness but emitted a strikingly lovely fresh, fruity aroma. .

It must be concluded from that extract yield and quality are not compromised when partitioning of the ethanol extract into non-polar solvents is undertaken after the buds have been removed. Indeed the modification to extraction protocol results in improvements for most parameters.

4.1.6. The Inclusion of Propylene Glycol in the Extracting Solvent.

Propylene glycol is used in many products as a non-toxic anti-freeze and as an inhibitor of fermentation and mold. In the experiments detailed in section 3.1.7 the chemical was incorporated to act as a 'keeper', preventing the loss of the highly volatile thiols in blackcurrant extract during RVE and as a humeticant to lower water activity, thereby reducing the potential for degradative reactions to occur. When propylene glycol was included in the extracting solvent the extracts were dark green to

black, with deep fruity aromas. Excess propylene glycol resulted in the separation of the solvent from the extract, producing an un-marketable product. Propylene glycol could not be removed by RVE under the conditions trialed. However, the aroma impact of propylene glycol extracts, assessed organoleptically following storage for long periods of time, were of significantly higher quality than the extract produced using the standard method.

4.1.7. Extraction of Blackcurrant Buds Using the Laboratory Based Method with the Inclusion of the Antioxidant BHA

Non-enzymatic oxidation in organic systems often begins with the removal of a hydrogen atom from a carbon adjacent to a double bond resulting in a free radical. The initial removal of hydrogen requires energy and so is influenced by storage temperature and is catalysed by light (CRC Handbood of Food Additives). Molecular oxygen reacts with the radical to produce an activated peroxide which is very reactive and requires very little energy to remove hydrogen from another carbon adjacent to a double bond. Antioxidants such as butylated hydoxyanisole (BHA) contribute hydrogen to stop the auto-oxidation process, themselves forming stable free radicals that do not propagate further radical formation. The extraction process to produce concrete exposes the components of blackcurrant buds to molecular oxygen. To retard oxidation processes an antioxidant (BHA) was included in the extracting solvent to reduce loss of components. The extraction undertaken was a two step process. An ethanol extract of buds was partitioned against 5 % hexane in petroleum ether then the buds were re-extracted with 10 % ethyl acetate in 5 % hexane in petroleum ether to produce the marc. The yields from the 2 stages were not combined, but analysed separately. Although the inclusion of BHA in the extracting solvent appeared to improve the yield of most volatile components the standard deviation indicated that the differences were not significant. Only slight differences in the relative composition of the extracts produced with and without antioxidant were noted over time. However an organoleptic assessment of samples stored over a long period of time and that had been extracted with the inclusion of BHA, retained a significantly more potent blackcurrant aroma than those without BHA.

4.1.8. Effects of Freezing Blackcurrant Buds in Ethanol.

After harvest blackcurrant buds are placed in a box with a plastic insert and frozen prior to extraction. The freezing of bud material can result in the fragmentation of the brittle bud structure, leading to leakage of cell constituents whilst the formation of ice crystals further disrupts intracellular membranes (Finkle, 1971). In section 3.2.2 it was reported that a decrease 15 % in volatiles from mechanically harvest blackcurrant buds occurred after freezing and storage beyond a 2 week period resulted in further depletion. Freeze-protective additives such as glycerol have been reported to reduce the availability of free water in the cells and reduce the formation of ice crystals at sub-zero temperatures (Fennema and Powrie, 1964; Rapatz and Luyet, 1968). As ethanol was the preliminary extracting solvent the potential benefits in reducing crystal formation by freezing blackcurrant buds steeped in this solvent was trailed in this study. In addition low molecular weight alcohols have been reported to reduce enzyme action (Tressel *et al.*, 1970). However, the immersion of blackcurrant buds in ethanol prior to freezing did not retard the loss of volatiles. There was no significant difference in yield and composition of the extracts produced from un-rolled and rolled buds that had been stored frozen in ethanol for a period of 2 months and compared to buds frozen un-rolled and dry. The ratio of ethanol to bud weight was 2:1. Buds are approximately 50 % water such that the concentration of water would have been in the order of 25 %. Ice formation would still have been prevalent and ethanol may not be as effective in reducing water activity as solvents such as glycerol and ethylene glycols. The similar yield and quality of the extracts from buds stored in ethanol and the buds frozen without solvent also indicates that the loss of volatiles by enzymatic activity is minimal. Buds that had been rolled and frozen whilst immersed in ethanol produced slightly higher extract yields but this was offset by a 22 % decrease in volatiles. The yield and quality of extract from buds left un-rolled and frozen in ethanol was not significantly to warrant the implementation of this process in commercial practices.

4.1.9. Adaptation of the New Blackcurrant Extraction Method to Commercial Operations

The blackcurrant product produced in Tasmania is a viscous green to yellow extract with crystallised acids accumulated at the base of the containing vessel. The effects of the cultivar, seasonal effects and the available nutrition within the year of cultivation all impact on the yield and composition of annual production. However the methods of extraction impact significantly on the quality of the product. The permuatations of solvent type, volume, order of solvent addition etc. that could be trailed were limitless and this study attempted to investigate some aspects of the many variations possible.

None the less a new method of extraction was established which included the initial steeping of buds in ethanol to retard enzymatic activity during masceration. Organoleptic assessment perceived that the ethanol based extracts had a more pungent catty aroma, reminiscent of the European product. Variations in the order of different steps in the extraction process and in the solvent composition were trailed and the results were used to modify the ethanol based extraction method. In the preceding sections the protection of potentially labile volatiles during extraction with the inclusion of humeticants and antioxidants was also investigated. With no significant improvement in extract quality, as measured by the levels of components using GC FID and GC FPD, the addition of nonendogenous protecting species was found not to be advantageous.

Adaptations of the ethanol based extraction method to industry required lower ethanol volumes and the omission of the step by which the ethanol extract volume was reduced by RVE, prior to partitioning of the polar extract into 5 % hexane in petroleum ether. In addition, the inclusion of water in the industrial processes would have precluded the effective recycling of ethanol. Industry also specified that a reduction in the volumes of petroleum ether would be an advantage. Experiments described in section 3.1.10.i were undertaken to determine the volumes of ethanol required to extract the majority of ethanol soluble components in blackcurrant buds. A minimum of 2 : 1 ethanol : bud weight was required to effectively cover the buds and it was shown that when combined with a second wash of 1 x 1 : 1 of ethanol : bud

weight, 83 % of ethanol soluble compounds were extracted (table 3.1.xvi). The recovery was considered acceptable as not all the residual compounds that would have come out in the third wash would be soluble in the petroleum ether layer in the subsequent partition step. In addition the buds were to be back washed with ethyl aetate/hexane/petroleum ether such that any important components not extracted in the ethanol would be recovered. In line with the specification set by industry, in terms of the expense and the capacity to deal with large volumes of ethanol, the results from section 3.1.10.i were used to adjust the laboratory based method from a single 1 x 4 :1 ethanol : bud weight wash to a 1 x 2 : 1wash, followed by a 1 x 1 :1 wash.

With the lower volumes integrated into the extraction methodology, the further reduction in ethanol extract by RVE was no longer needed to ensure the formation of a distinct partition. Indeed experiments conducted showed that a distinct partition was formed using the new ethanol volumes without the addition of water (section 3.1.10.ii). The experiment also investigated whether the partition was distinct when ethanol volumes were the same as in the original method, but without the use of RVE to reduce extract volume. The partition without the volume reduction step and with water added was slow to form and the line of separation was hard to distinguish. In contrast the modified extraction process using a 1 x 2 : 1 and a 1 x 1 : 1 ethanol : bud weight ratio in the initial extraction, did not require a dry down step or the addition of water, to effect a distinct partition.

Included in the modifications to adapt the laboratory based extraction method to industrial scale included the reduction in the volumes of non-polar solvents to three extractions using 0.75 : 1 of 5 % hexane in petroleum ether relative to bud weight. In addition the marc was extracted with 3 x 1 : 1 10 % ethyl acetate in 5 % hexane in petroleum ether to bud ratio instead of the 1 x 4 : 1 ratio used in the laboratory based method. The effectiveness of these adaptions were assessed by sub-sampling fractions from each stage of the modified method. The results presented in table 3.1.xvii show that a fourth partition with 5 % hexane in petroleum ether may increase the recovery of oxygenatated sesequiterpenes, an advantage that would be offset by the likely increase

in acids that may be co-extracted. Only 20 % of acids present in the ethanol extracts was solvated into the 3 partitions of 5 % hexane in petroleum ether undertaken.

Experiments were established to compare the original laboratory based method with the method modified for industry. It was found that extract yield was reduced by 17 % using the modified method, however this was offset by the reduction in acids by almost 50 % (table 3.1.xviii). The extract also had lower levels of non-volatiles and late eluting components of lower volatility.

The concretes produced using the original extraction methodology were identified as having the most 'catty' quality and possessing an overall higher aroma quality when assessed organoleptically. This corresponded to higher levels of the endogenous thiol which was found to be in the order of 6 % higher (table 3.1.xvii). The new methodology presented to industry is summarised below.

Blackcurrant Extraction Protocol - Commercial Adaptation

1. Roll frozen buds and immerse in 2 : 1 ratio of ethanol to buds
2. Agitate (tumble) for 2 hours remove solvent
3. Re-extract buds in 1 : 1 ratio of ethanol to buds and agitate for 2 hours
4. Add 5 % hexane in petroleum ether at a ratio of 0.75 : 1 relative to bud weight
5. Agitate and allow phases to separate then collect the non-polar fraction

(Note; phase separations were very slow to form in the industrial trials)

6. Repeat steps 4 and 5 twice.
7. Re-extract the buds with 1 : 1 ratio of 10 % ethyl acetate in 5 % hexane in petroleum ether relative bud weight.
8. Agitate for 2 hours and remove the solvent
9. Repeat steps 7 and 8 a further 2 times.
10. Combine the marc extract with the non-polar partition fractions and dry down using RVE at 40°C.

This method has since been trialed by industry. Difficulties in recovering extract were encountered and polymerisation of the final product was evident after a period of storage. None the less the product was accepted by the market. The method presented forms a viable framework from which continual improvements and modifications can be investigated at the industrial level.

4.1.10. Stability of Endogenous Thiols in Blackcurrant Extracts

Thiols are sulphur analogues of alcohol and are easily oxidised and converted into disulphide –S-S- links. They form the same kind of derivatives as alcohols such as thio-ethers, thio-acetals and thiol esters (Morrison and Boyd, 1987). Experiments detailed in section 3.1.11 showed that the thiol endogenous to blackcurrant dissipates rapidly in extracts with only 15 % detectable in samples stored for less than a month. This corresponded with the findings of (Hofmann *et al.* 1996) who undertook model studies into the oxidation of thiols which contribute to the odour of roasted coffee and bread. The effects of the solvent on synthesised compounds were most significant in diethyl ether with 20 % of the most labile thiol investigated 2-methyl-3-furanthiol (MFT) converted to MFT-MFT after 1 day of storage. After 10 days of storage only 50 % remained. Solvation in dichloromethane reduced oxidation rates to 6 % after 9 days whilst in n-pentane MFT was most stable as no significant oxidation was observed. Heating catalysed oxidation in all solvents.

The loss of between 6 and 9 % of 4-methoxy-2-methyl-2-butanethiol in blackcurrant extracts (section 3.1.11.i) after 1 day with between only 60 and 70 % remaining after 8 days confirms the lability of the thiol endogenous to blackcurrants. In reference to Eiserich, 1994, Hofmann proposes that the activity of MFT is based on the easy abstraction of a hydrogen atom from the thiol group with the intermediate formation of a thiyl radical being stabilised by electron delocalisation. Although 4-methoxy-2-methyl-2-butanethiol does not possess a methylated furan ring to allow for such delocalisation the tertiary nature of the thiol wqould provide for some delocalisation of electrons to stabilise an intermediate radical, promoting oxidation. Methods were trialed to investigate whether the inclusion of antioxidants, humeticants or the use of alternative extraction methods may protect the thiol concentration. Using the standard

extraction method (5 % hexane in petroleum ether), a loss of 45 % of the thiols within 21 days occurred and the addition of protecting agents such as BHA and propylene glycol did little to slow the rate of depletion (table 3.1.xxii, page 106). This corresponded with the findings of Hofmann 1996 who found that the oxidation of MFT dissolved in diethyl ether was not inhibited by the antioxidant butylated hydroxy toluene. However, despite the lack of chemical evidence to the preservative properties of the antioxidant BHA, the following should be noted. In the experiment conducted in 1998, where the antioxidant was included in the extracting solvent (section 3.9), the resulting concrete retained potent blackcurrant aroma for over five years when compared to concrete produced without BHA.

Unfortunately the samples from ethanol based extraction method were not easily solvated in the solvent used for GC analyses such that the results over the first two weeks of storage were un-reliable. However the results indicate that the thiol may be retained over some period of time in concrete produced using this method. This is also supported by the results obtained for the ethanol-based extractions reported in the antioxidant experiment (section 3.9). Even without antioxidant, 80 % of the thiol remained in the concrete after 26 days. This is significantly higher than the 57 % of thiol remaining in the control after 21 days in the experiment described above. Although the majority of the solvent is removed, the varying propensity for oxidation of thiols in the presence of different solvents may account for the differing rates of depletion. The loss of this important aromatic volatile is an aspect in the production of blackcurrant extract that must be addressed by continued research.

Section 4.2. DORMANCY, FREEZING AND INCUBATION

4.2.1. Variation During Dormancy of the Chemical Composition of Blackcurrant Buds

It has been reported that blackcurrant oil gland size increases at the time of rapid leaf growth, maximising when photosynthate can be redirected from leaf growth to secondary metabolism (Kerslake, 1984). Indeed the enzymes of terpene biosynthesis are more active in young rapidly growing tissue than in mature tissue (Croteau *et al.*, 1981; Gershenzon and Croteau, 1991). The rate of oil accumulation declined in

blackcurrants in autumn as the average daily incident of solar energy declined (Poulter, 1991). However once a terpenoid pool is accumulated there is little evidence that substantial quantities of terpenes are lost as a result of metabolic turnover, volatilisation or leaching (Gershenzon, 1994). Yet in this study the dry weight of buds decreased throughout dormancy at the commercial plantation and for cv. White Bud and HTC at the Cambridge farm, by 18, 14 and 16 % respectively, with a corresponding decrease in yield of volatiles of 29, 27 and 24 % respectively (section 3.2.1). Similarly the yield of volatile components also decreased throughout the dormant period. This is not in line with the constant yields of concretes extract from buds through dormancy reported by Poulter in un-published data (1992).

Terpenoids are more expensive to biosynthesise per gram in terms of plant resources than most other primary and secondary metabolites due to their extensive chemical reduction (Gershenzon, 1994). Terpenoid accumulation is offset by the benefits such as protection against herbivores, though when dormancy proceeds without disruption from predators such as insects or mites much or the resources remain under-utilised. In the absence of sexual organs the attraction of entomophages amd pollinators, or the action of allelopathy, precludes loss by increased emission of volatiles. Accumulated terpenoids may be catabolized in times of low photosynthate availability (Loomis and Croteau, 1980). Evidence as to degradation of monoterpenes for carbon reutilisation and to a lesser extent, energy generation has been reviewed (Croteau, 1987). The loss of menthone in peppermint leaves of between 70 to 80 % reported by Croteau far exceeds the 20 % loss of volatiles recorded in this study and the decrease was fairly uniform across all the classes of terpenes monitored. The catabolic pathways by which terpenoids are oxidised for carbon re-utilisation and energy generation are specific and yet no one terpene dissipated at markedly higher rates. Volatile dissipation then is probably not due to scavangening but simply through passive volatilisation and leaching at a time of low photosynthate availability that limits the continued maintenance of terpenoid levels through dormancy.

The level of endogenous thiol detected in the selected clones decreased by 36 % though this contrasted with the levels in cv. White Bud which increased by over 50 %

at site 1 but remained relatively constant at site 2. As a broad overview, however, the decrease in bud weight and yield of components for all blackcurrant buds sampled in this study would suggest that the harvesting of buds at the end of the dormancy is not necessarily optimal. Indeed the level of acids extracted increased late in dormancy and as bud burst proceeded. Although the quantitative results indicate that there is no apparent advantage to harvesting before the end of dormancy to harvest, the beginning of bud burst may provide for increased bud yields. At the final sampling at site 1, 23 % of buds had burst and although the degree of bud burst at site 2 on the final collection date was not recorded the % dry weight of buds and the % yield of volatiles had increased markedly at both sites. The quality of the oil in this regard is the foremost consideration. The aroma impact of extracts, however, was not assessed in this study as blackcurrant concretes were not produced. Kerslake, (1984), reported a 167 % increase in bud yield from 0 to 94 % bud burst. Organoleptic comparisons of extracts of buds collected by Kerslake, (1984) over the period of bud opening, found that the catty note, attributed to the natural thiol, increased as bud burst progressed. He reported that the quality and background fruit impression of the blackcurrant extracts produced remained well balanced up until 50 % bud burst. Thereafter the catty note was found to overwhelm the blackcurrant aroma. The determination of optimum harvest date for maximum yield and quality must therefore take into consideration commercial advantages of harvesting at the onset of dormancy, prior to depletion of bud components, or harvesting after bud burst has commenced but prior to the opening of 50 % of the buds.

This study has also served to quantify the differences between the cv. White Bud and the HTC. Clones, propagated from selected high thiol-containing cv. White Bud stock produced buds of higher yield and quality. The levels of sabinene and bicyclogermacrene in extracts of HTC were significantly higher than in cv. White Bud, although those high levels fell during dormancy. Relative concentrations of sabinene, δ-3-carene, limonene, β-phellandrene and terpinolene have been used to distinguish varieties (Latrasse and Lantin, 1974). Using the premise established by Latrasse the high levels of sabinene in the HTC preclude any relationship between this selection and the families Noir de Bourgogne, Royal de Naples or Brodtorp whilst the

presence of δ-3-carnene further preclude the families Mendip cross, Cotswold cross, Tor cross, Malvern cross and Golubhka. Although Latrasse's key places the HTC selections in the same family as Baldwin, from which White Bud is a selection, none the less HTC differs from White Bud in the relative levels of all monoterpenes used to distinguish the families to the order of 1:3, 1.6:1, 11.5:1 and 1.6:1 for sabinene, δ-3-carene, limonene/β-phellandrene (co-eluted) and terpinolene respectively. No direct correlation between the quantities calculated in this study and the figures recorded by Latrasse can be drawn as the extraction protocols differ markedly. A more detailed study was published investigating the chemotaxonomy of 23 blackcurrant cultivars of diverse parentage and origin by relating the abundance of six monoterpenes, eight oxygenated monoterpenes, seven sesquiterpenes and two oxygenated sesquiterpenes extracted from blackcurrant buds (Latrasse *et al.*, 1990). Inter-relating the relative abundance of the monoterpenes of the two selections from this study using Latrasse's chemotaxonomical formulations again confirmed the close relationship of the HTC and White Bud to the cultivars with Baldwin parentage whilst clear distinctions in the profiles negated genetic connections with the cultivars originating from France. In a study comparing 11 cultivars originating from France and Australia, White Bud was again distinguished by relative abundances of sabinene, δ-3-carne, α-terpinolene and β-pinene (Kerslake *et al.*, 1989). Levels of these components quantified in the study reported here within were to the same order in HTC and White Bud however sabinene was at levels more than 3 fold higher in HTC buds. The sesquiterpenes used to discriminate between varieties in Kerslake's study were γ-elemene, α-humulene, germacrene D and 2 unknown components. Quantification of only α-humulene and germacrene D was undertaken in this study and the ratios of these components in White Bud relative to HTC were 2:1 and 2:0 respectively. The sesquiterpenes quantified in the Latrasse's study (1990) included β-caryophyllene, α-humulene, germacrene D and bicyclogermacrene as well as the oxygenated sesqiterpene caryophyllene oxide. The relative abundance of β-caryophyllene and α-humulene and caryophyllene oxide in HTC and White Bud are similar with ratios of White Bud is to HTC of 1.6:1, 2:1 and 1:1.6 respectively. Caryophyllene oxide and other oxygenated C_{15} chemicals are considered genetic markers because of their route of formation

(Latrasse *et al.*,1990). However, the total absence of germacrene D in HTC, along with a 10 fold higher level of bicyclogermacrene is reminiscent of cultivars with French parentage. Yet the HTC were propagated from selections of White Bud and the differences are not of sufficient magnitude to contradict the conclusions of Kerslake (1985) that White Bud is a local selection of Baldwin. However as the terpenoid profile of blackcurrant has been shown to be consistent across cultivars and is genetically related, the deviation from the standard profile exhibited by HTC may not be attributed to cross pollination and/or the expression of the combination of two recessive alleles as the blackcurrants are propagated from cuttings. In this case a level mutation may have occurred.

Interestingly, the high production of sabinene and bicyclogermacrene within HTC blackcurrant buds seems to be matched by an equal propensity for their metabolism through dormancy.

In summary then, HTC differ from the cv. White Bud variety of blackcurrants by containing higher levels of 4-methoxy-2-methyl-2-butanethiol, sabinene, myrcene, bicyclogermacrene and hardwickic acid in bud extracts. Conversely, cv. White Bud buds had significantly higher levels of β-caryophyllene, β-phellandrene and limonene, the latter two of which co-eluted under the GC conditions used and so were indistinguishable by FID analyses. This study found the relative levels of sabinene to be a distinguishing parameter. The proportion of sabinene in the monoterpene fraction ranged from 19.0 to 17.5 % throughout dormancy in the cv. White Bud, compared to a range of 54.1 to 54.4 % in HTC. δ–3-Carene and terpinolene were not discriminating components and their paired presence is in affirmation that these and the pair limonene and β-phellandrene are either both present or both absent (Latrasse and Lantin, 1976). Bicyclogermacrene was also markedly higher in the selected clones. This component has been found to be particularly abundant in strongly aromatic varieties Noir de Bourgogne (Le Quéré and Latrasse, 1990).

4.2.2. Effect of Freezing of Commercially Harvested Buds

The limitations of commercial operations necessitates that blackcurrant buds be frozen after harvest until sufficient quantities are accumulated for large-scale batch

extractions. However within 24 hours of post-harvest freezing over 50 % of the naturally occurring thiol, 4-methoxy-2-methyl-2-butanethiol is lost as are 18 % of volatile components. Longer term freezing resulted in a steady overall loss of volatile components. Freezing reduces reaction rates and bacterial growth, often deactivating enzyme systems and hindering the circulation of reactants and gases in tissues (Finkle, 1971). Associated deleterious changes include damage to cellular structure and leakage of cell constituents. The release of cell constituents allow for enzymatic and non-enzymatic reactions to occur that may deplete volatiles through catabolism and oxidation. Although tissues are relatively stable to storage once frozen, chemical and enzymatic reactions are still active. The continued loss of volatiles during storage may result from continued enzyme activity which can proceed at temperatures below -120°C (Bielske and Freed, 1965) and indeed reaction rates can increase in frozen solutions (Grant and Alburn, 1966; Kiovsky and Pincock, 1966).

Temperature and rate of freezing and displacement of cellular water are believed to be major factors in the freezing damage. The most damaging region of temperature is near -10°C to -20°C where large crystals of ice are formed. Structural integrity is maintained at low rates of cooling in the order of 1 to 10°Cmin^{-1} (sub-reference Finkle 1971). The loss of volatiles observed when blackcurrant buds are frozen may be reduced if controlled rates of freezing are introduced and the freezing temperature lowered to below -20°C.

4.2.3. Effect of Mechanical Damage and Incubation on Volatiles in Commercial and Clonal Buds.

4.2.3.i. Effect of damage from machine harvesting on thiol content and post-harvest thiol synthesis in blackcurrant buds.

The concentration of 4-methoxy-2-methyl-2-butanethiol is maintained at varying levels within blackcurrant buds, however, it must be considered that the susceptibility of thiols to oxidation and dimerisation is increased when buds are harvested and the cellular compartments deteriorate. When blackcurrant buds are hand-cut from the cane, minimal damage to the structure is incurred. The reduced exposure to oxidative conditions may account for higher thiol concentration (4.6 mgkg^{-1} DMB) detected in

intact buds compared to those extracted from machine-harvested material (3.5 $mgkg^{-1}$ DMB, section 3.2.3). The oil producing glands are found in increasing density on the inner structures of the bud and rows are present on the base of each bud layer (Poulter, 1992). Some buds are located short pedicels. During harvest many of these buds are only partially removed thus some of the oil bearing structures remain attached. This is most likely increased in machine harvesting and may account for some of the loss of thiols and volatiles.

The poorer yields of most components in machine-harvested buds may also be due to a dilution effect. The thiol concentration is calculated relative to the dry weight of buds. Machine harvesting results in the inclusion of a larger amount of bark and stem material, thereby diluting the concentration of thiols in the material extracted. Distinguishing this effect from the loss of thiols due to damage by machine harvesting was problematic as the collection of smaller plant fragments could not be established as deriving from buds or from co-harvested debris.

Post-harvest thiol production was most rapid in fresh hand-cut HTC buds incubated at 10°C in air resulting in a 200 % increase after 50 hours. This is the first reported post-harvest increase in thiol concentration. Whether post-harvest increases are due to synthetic or degradative processes was not differentiated in this study but the presence of non-aromatic cysteinylated precursors may have provided for the release of the volatile thiol. The release of sulphur-containing compounds such as 4-mercapto-4-methylpentant-2-one from odourless precursor fractions of Sauvignon grape juice by alcoholic fermentation has been reported (Darriet *et al.*, 1993). Indeed none of the thiols which confer blackcurrant and boxtree nuances to wines have been detected in non-fermented musts (grape juice). As the retention of bud structure resulting from hand harvesting provides for maximum post-harvest thiol formation it may be postulated that the enzymatic cleavage of the thiol from non-volatile precursors accounts for increased levels rather than indiscriminate oxidative and degradative processes. Mechanical harvesting of HTC buds resulted in the initial loss of 18 % of thiols and reduced post-harvest thiol production. Similarly 25 % of thiols were lost within 24 hours in machine harvested commercial buds and despite some thiol

production in fresh machine harvested buds over 72 hours, post-harvest synthesis declined thereafter. The detrimental effect of structural damage to post harvest thiol synthesis and accumulation in buds was further demonstrated by the reduction in thiol concentration recorded when machine-harvested commercial buds were further damaged by rolling. This reduced thiol levels by 41 % and, within 24 hours, levels had further decreased to below detection level. The release of thiols from cysteine precursors has been catalysed by cysteine-S-conjugate β-lyase (Tominaga *et al.*, 1998b;Wakabayashi *et al.*, 2002). Indeed the α,β-elimination reaction of S-cysteine conjugates catalysed by tryptophanase to enzymatically release thiols have been used to assay for the aromatic potential of *Vitis Vinifera* L. cv Sauvinon Blanc grapes and Cabernet Sauvignon and Merlot musts (grape juices) (Peyrot des Gachons *et al.*, 2000; Murat *et al.*, 2001). The fermentation of grape juice is, naturally, fundamental to the production of wine. The application of fermentation and the introduction of cysteine-S-conjugate lyases into blackcurrant extracts would further elucidate the process involved in the bio-synthesis of thiols. The post-harvest production of thiols in blackcurrants was suppressed under a nitrogen atmosphere. This is consistent with the altered composition of emitted volatiles from harvested flowers purged with nitrogen compared to air purged flowers (Mookherjee *et al.*, 1986; Mookherjee *et al.*, 1989; Mactavish and Menary, 1998a). Both enzymatic and oxidative processes would be suppressed when air is limited. Further experimentation to include enzyme inhibitors would provide further information into the processes involved in post-harvest thiol synthesis.

4.2.3.ii. Effect of Freezing Damage on the Post-harvest Synthesis of Thiols.
Freezing commercial buds reduced thiol content by 35 %. Post-harvest thiol production in defrosted buds that were incubation at 10°C was similar to that recorded in fresh buds. However, thiol production ceased after 72 hours in incubated fresh buds, whilst production in frozen-thawed buds continued for up to 168. This may be related to the availability of substrates by decompartmentalisation of fluids within freeze damaged cells and indicates that some form of damage is required to allow for post-harvest thiol synthesis.

4.2.3.iii. Effect of Mechanical Damage on the Post-harvest Synthesis of Volatiles

Oil glands constitute the major sites of monoterpene biosynthesis and accumulation (Croteau, 1984). Many terpenes are present as non-volatile constituents and it has been found that glycosidically bound flavours can exceed the amount of the free aroma in a ratio range of 2:1 to 5:1. Aromatic volatile compounds bound to glycosides can be released during storage, pre-treatment or by enzyme and acid catalysed reactions (Crouzet and Chassagne, 1999). Following harvest the production of terpenoids can continue. The post-harvest production of volatiles is evidenced in hand-cut, fresh blackcurrant buds (section 3.2.3.iii) and may result from the release of aglycones from glycoconjugated tepenoids or from degradation and rearrangement of terpenoids already present in the bud. Despite an initial loss of 13 % over 16 hours, volatile concentrations rose above the levels detected prior to incubation within 48 hours and were maintained for over 72 hours in hand-cut HTC buds. A nitrogen atmosphere only had a minimal effect on the suppression of volatile production. However when fresh HTC buds were mechanically harvested and incubated no post-harvest volatile synthesis was evident and levels decreased by 19 % within 16 hours. It must be postulated that the loss of post-harvest volatile production is due to the damage to the structure of the bud incurred by the mechanised harvester. Minimising damage to structure resulted in maximum post-harvest synthesis. The retention of compartmentalisation had either contributed to reduced metabolism and oxidation of existing terpenoids or retained the viability of enzymatic processes.

Incubations of hand-cut and machine-harvested HTC buds were continued for 72 hours. No volatile production was recorded in machine-harvested HTC buds. However, when machine-harvested, un-rolled fresh buds (cv White Bud) were incubated post-harvest volatile production was clearly evident and levels increased by 28 % after 20 days at 10°C. The un-rolled frozen mechanically harvested buds (cv White Bud) showed a similar trend. Despite the overall increase in volatiles in the fresh buds (cv White Bud), however, a decline was recorded for the first 2 days of incubation. The incubation of fresh machine-harvested HTC buds was only undertaken for 3 days. It is difficult to accept that HTC buds, which are a selection of White Bud, are more susceptible to mechanical damage in terms of post-harvest

production of volatiles than cv White Bud. Perhaps if the incubation of the HTC buds had continued beyond 3 days an increase in volatiles may have been recorded. In the absence of this data the differences cannot be explained.

The detrimental effects of damage to bud structure on post-harvest volatile production is more clearly evidenced in the incubations of mechanically harvested, rolled and un-rolled commercial buds. Increased damage to buds caused by rolling resulted in a dramatic depletion of volatiles and had a significantly greater detrimental effect in the retardation of post-harvest production than did freezing. Fresh and previously frozen, un-rolled buds produced an increase in volatiles during incubation at 10°C whilst rolling buds caused an immediate loss of volatiles and an arrest of all post-harvest synthesis. The effect of structural damage on extract quality has previously been encountered. The post-harvest treatments of chopping and storage of coriander herb prior to extractions resulted in lower levels of aldehyde and concomitant increases in the relative levels of alcohols (Smallfield *et al.*, 1994). Un-chopped coriander could be stored for 24 hours before distillation with no effect on oil composition. Smallfield, (1994) speculated that the change in oil chemistry triggered by chopping was probably due to the release of an enzyme stored separately from the oil glands which reduced the aldehydes to the corresponding alcohols. The formation of the alcohols was likened to that reported in several plant species and attributed to oxidoreductase activity (Schreier, 1984). Aldehydes are not a major constituent of blackcurrant extracts but the marked difference in the relative levels of the monoterpenes, sesquiterpenes, oxygenated sesquiterpenes and acids indicate the processes involved in the change of profiles effected by damage to buds post-harvest operates by significantly different processes across these four classes or chemicals. For the monoterpenes, typified by the levels of α-pinene, post-harvest synthesis was evident only in un-damaged buds. Structural damage, and by inference, decompartmentalisation of substrates, had de-activated enzymatic processes leading to monoterpene synthesis. In addition, monoterpenes are highly volatile and crushing of the bud structure would enhance loss by volatilisation. A similar decrease in sesquiterpene concentration in rolled buds occured at a slower rate than that recorded for monoterpenes over the incubation period and this may be related to the lower

volatility of the higher molecular weight C_{15} chemicals. The overall increase in α-pinene in un-damaged buds was consistent with the increase in concentration of α-pinene and β-pinene recorded in boronia flowers (*Boronia megastigma* Nees) in air-purged flowers incubated at 25°C for 24 hours, a process inhibited to a small extent in a nitrogen atmosphere (Mactavish and Menary, 1998a). The increase in free volatiles in boronia flowers after harvest occurred only in fresh, undamaged flowers, strengthening the postulation by MacTavish and Menary, (1998a) that the changes are attributable to enzymatic activity.

The levels of the sesquiterpene, β-caryophyllene, were also increased only in un-rolled bud material. This pattern of structural damage inhibiting all post-harvest synthesis was not evident, however, in oxygenated sesquiterpenes such as caryphyllene oxide and in hardwickic acid. Indeed post-harvest production in rolled buds effected increases to the same order as that observed in intact buds. The disparities in the behaviour of the post-harvest production across the different terpenoid families are consistent with the sub-cellular compartmentalisation and spatial regulation proposed as a significant element in terpenoid metabolism (McGarvey and Croteau, 1995). The main intra-cellular site for biosynthesis of the higher molecular weight terpenoids may well be removed from that of monoterpenes. The de-activation by freezing of processes which increased the levels of oxygenated sesquiterpenes and terpene acids was confirmed by the constant levels maintained for both components when incubated at 10°C following a freezing event. Rolled buds returned slightly higher levels, perhaps as a result of improved solvent penetration into the inner layers of the bud which contain significant densities of oil glands (Poulter, 1992). It may be postulated that the post-harvest synthesis is via unregulated enzymatic activity and metabolism of related components by processes that are not reliant on continued compartmentalisation of reactants. These processes along with oxidative degradation and rearrangement were de-activated by freezing.

4.2.3.iv. Effect of Freezing Damage on the Post-harvest Synthesis of Volatiles

As discussed, freezing of machine-harvested buds before incubation at 10°C for 72 hours had only a slight detrimental effect on post-harvest production of monoterpenes

and sesquiterpenes compared to the effect of structural damage caused by rolling. It has been established in the previous section that maximum post-harvest production of volatiles was recorded in HTC buds that had been harvested by hand (figure 3.2.ix). Yet post-harvest synthesis of volatiles was absent in these, hand-cut HTC buds when frozen prior to incubation (figure 3.2.xi). In contrast machine-harvesting halted volatile production in fresh hand-cut buds yet volatile concentration increased when machine-harvested and frozen prior to incubation. Un-damaged bud structure retains the compartmentalisation required for many metabolic and catabolic processes. Freezing has the effect of de-activating many enzymatic processes whilst the formation of ice crystals can disrupt organelle structure and cause leakage of compartmentalised components (Finkle, 1971). The processes involved in the post-harvest production of volatiles in fresh buds, then may differ from those active in freeze-thawed samples. That is, that the residual systems still functioning in fresh-hand cut buds may still be facilitating the enzymatic production of volatiles. In frozen buds, however, many enzyme systems may be de-activated such that volatiles may be instead produced by the interactions of precursors, enzymes and oxidative elements freed from compartmentalisation by damage to bud structure by freezing. Unfortunately the incubations of previously frozen clonal buds were not continued long enough to indicate whether factors such as exhaustion of substrates released by freezing damage would eventually lower the level of volatile production.

Post-harvest production of different classes of components, constituting the volatile fraction, are affected in different ways by post-harvest conditions. As discussed in the previous section monterpene levels dropped within the first 24 hours of all incubations but post-harvest synthesis occurred in un-rolled buds thereafter. As post-harvest synthesis was absent in both fresh and frozen rolled buds damage to bud structure has a greater detrimental effect on the level of α-pinene than does freezing. As freezing occurs, water crystallisation can affect substrate concentration and alter pH and ionic equilibria. However, this did not inactivate the processes of post-harvest production of extract components. The rolling of buds can effect similar changes but the increase in surface area, affected by gross structural damage, would increase the potential for oxidation and volatilisation of components. For components with higher molecular

weights, such as caryophyllene oxide and hardwickic acid, the freezing of buds had a more pronounced effect on post-harvest synthesis than did rolling. Although freezing did enhance the amount of caryophyllene oxide extracted at the beginning of incubations, possibly as a result of oxidative processes, post-harvest synthesis was de-activated in frozen-thawed commercial buds. This loss of post-harvest synthesis of caryophyllene oxide and hardwickic acid by freezing indicates that the production evident in fresh material, may be due to enzymatic pathways which are vulnerable to low temperatures, or that the release of substrates from the confines of cellular structures damaged by ice crystallisation, may extinguish non-enzymatic synthesis.

To summarise the experiments detailed in section 3.2 and 4.2, biological materials are frozen to retard enzymatic and non-enzymatic processes, thereby preserving the quality of products. However, freezing also has the potential to activate enzyme systems. As water freezes the solutes in the remaining liquid become more concentrated, the pH changes and cell injury by ice crystals can cause the leakage of cell contents, facilitating interaction of enzymes and substrates (Lovern and Olley, 1962). In addition, autolytic and microbial activity, promoted by freezing and structural damage associated with commercial harvests, can result in significant changes in extract composition. This study attempted to determine the effects of these processes on the components of blackcurrant buds and the results indicate that there is the potential to manipulate the degree of damage incurred during the harvesting programs, and to vary the conditions under which the buds are stored, to enhance blackcurrant oil yield and quality. The retention of bud structure, effected by cutting buds from canes using a scalpel, presents the potential to increase thiol and other volatile levels by as much as 2.2 and 1.13 fold, respectively, in post-harvest incubations of fresh clonal buds. This potential is reduced when buds are partially damaged by machine harvesting and, in commercial buds, is lost when further damage is incurred by crushing the buds in a mechanised roller. Freezing of the buds may cause an initial loss of as much as 54 and 18 % of thiols and volatiles, respectively. Yet the combination of machine harvest damage and freezing can provide for improved oil quality. Although hand-cut, fresh buds will produce the highest level of post-harvest synthesis of volatiles, freezing intact buds retards most post-harvest

synthesis, indicating that some degree of damage is required if incubations are to be undertaken on frozen buds. In addition, volatile production in both fresh and previously frozen, intact buds declines after 72 hours, which may be a result of depletion of substrates within the intact organelles. In machine harvested buds, frozen prior to incubation, thiols and volatiles continue to be produced up until 168 hours, and production may have continued longer had the incubations been prolonged. More significantly, however, freezing of buds prior to incubation has the potential to increase the level of monoterpenes and sesquiterpenes whilst completely retarding the continued production of sesquiterpene oxides and diterpene acids. The degree of damage rendered on blackcurrant buds, instigated in conjunction with freezing and incubations, can be manipulated to meet the qualities specific to the end use of the product.

4.2.4. Pilot-scale Incubation of Blackcurrant Buds for Improved Extract Quality and Yield.

In all the experiments detailed in sections 3.2.1 through to sections 3.2.3, composition of volatiles in blackcurrant buds through dormancy and in buds that are incubated prior to extraction, were not assessed for aroma characteristics. The relationships between chemical profiles of blackcurrant concrete as detected by GC and the aroma impact are not necessarily direct. Many highly odorous chemicals may be present at levels below the detection limit of the analytical methods employed yet have extremely low aroma threshold values. In addition combinations of aromatic volatiles act synergistically to alter significantly the overall impression. The pilot-scale harvest and incubation experiments described in section 3.2.4 were to facilitate the production of blackcurrant concretes from buds harvested at different times through dormancy and subject to incubation. Extraction protocols replicated as close as practicable, the methods employed by industry.

Many of the results observed in the laboratory scale experiments were confirmed in the results obtained in the pilot-scale trials, although many disparities were evident between the two seasons. Figure 4.2.i. extracts the results listed in table 3.2.vi for the oil and thiol %yields (DMB) for the harvest years 2001 and 2002 and presents them

as histograms for ease of interpretation. The oil yield and level of thiols have been selected for presentation as both are fundamental to improving the yield and quality of blackcurrants. Again for the purpose of easy reference in this discussion histograms for the four components α-pinene, β-caryophyllene, caryophyllene oxide and hardwickic are presented as being representative of the changes recorded for the four major classes of terpenoids, those being monoterpenes, sesquiterpenes, oxygenated sesquiterpenes and acids respectively.

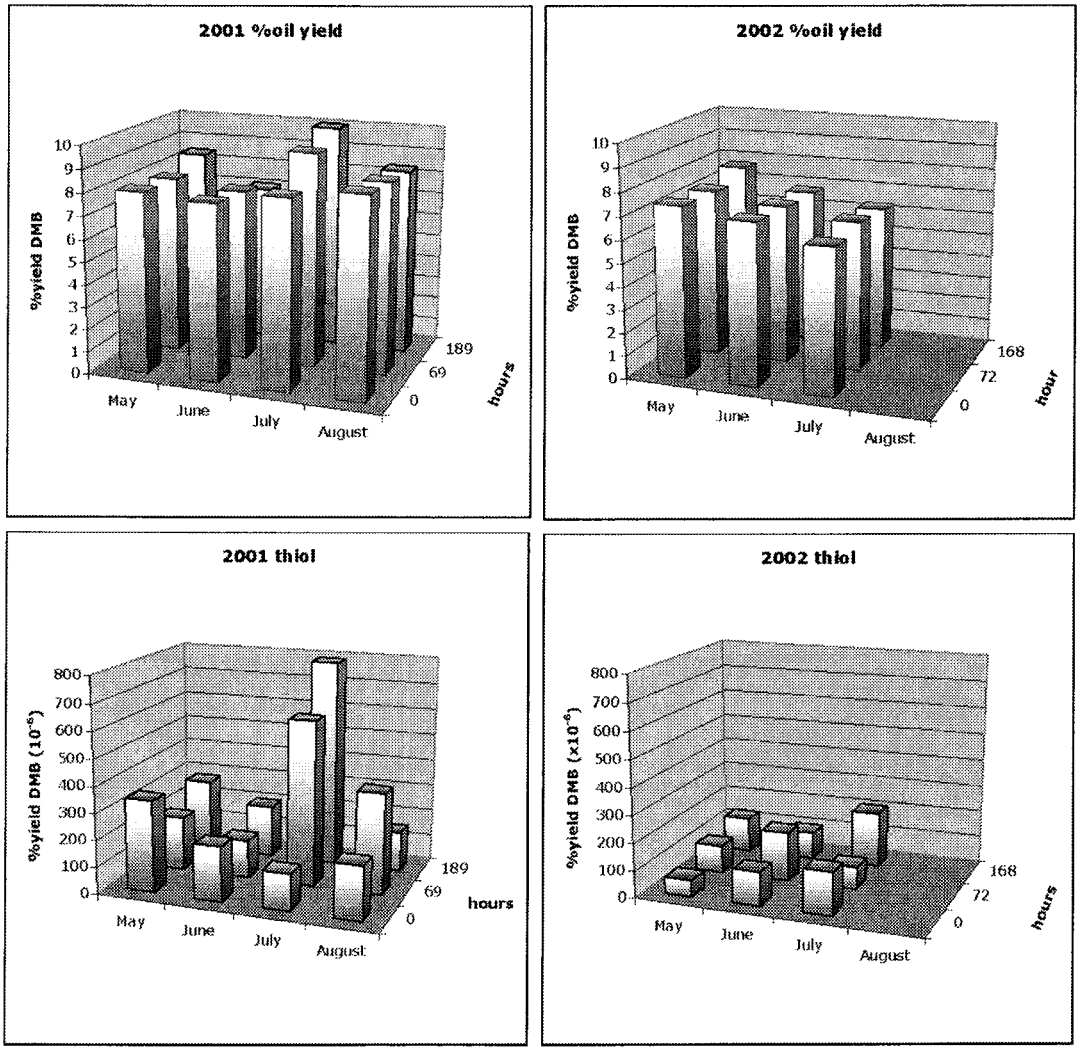


Figure 4.2.i. Histograms Presenting the %Yields of Oil & 4-Methoxy-2-methyl-2-butanethiol Harvested at Monthly Intervals and Incubated at 10°C.

During dormancy it would be expected that the requirements for photosynthate would be minimal yet, as observed in the laboratory scale harvest and incubation experiments

and in the above figure, the yield of oil decreased from May up until immediately prior to bud burst. The loss, however, did not exceed 15 % and may be attributed to volatilisation and leaching. The increase in oil yield observed in the 2001 harvest immediately prior to bud burst reflected the activation of metabolic processes towards leaf development and this naturally would require photosynthate. The fluctuations in the levels of δ-3-carene and terpinolene was suggested as evidence of metabolic turnover in blackcurrant buds during bud burst by Kerslake, 1984. Kerslake identified the increase in limonene with the concomitant decrease in α-terpineol and the subsequent reversal of the relative levels as indication of the switch from catabolism to photosynthetic processes as the source of carbon. Kerslake monitored levels weekly through the period of bud burst. The scale of the extractions undertaken in this study precluded such closely spaced monitoring. However the yield of the endogenous thiol and other components were monitored monthly and in the 2001 harvest, closely followed the trends reported by Kerlake. Levels of 4-methoxy-2-methyl-2-butanethiol decreased, or remained relatively constant, up until bud burst in August as metabolic pathways were activated. In 2002, however, thiol yield increased in non-incubated buds from May through until July. Indeed the benefits of incubation are also evident one month earlier in 2002 compared to 2001. The harvest year 2002 in Southern Tasmania had an exceptionally mild winter relative to the preceding years. Table 4.2.i. present the degree days above a base temperature of 5°C (pers. comm.) for the years 1999 to 2002 recorded at an automatic weather station located close to the sites of the blackcurrant plantations where both trials were conducted. The cumulative degree days are calculated as follows

$$\text{degree days / month} = \sum \frac{(\text{daily max temp} - \text{daily min temp})}{2} - 5°C$$

year	April	May	June	July	August
1998	211.8	174.0	131.3	141.5	129.0
1999	222.5	188.5	159.0	164.5	182.5
2000	287.0	216.0	164.0	170.0	175.5
2001	265.1	188.0	185.5	165.0	156.5
2002	295.5	234.5	198.0	179.0	186.5

Table 4.2.i. Cumulative Growth Temperatures (°C) at Precinct of Harvest and Incubation Trial Sites for the Relevant Months from 1998 to 2002.

Table 4.2.i demonstrates that the year 2002 had higher cumulative growth temperatures than the four preceding years for months in which the harvest trials were conducted. This may account for the disparities in the harvest month in which incubations were most effective as well as explain why the levels of thiol increased a month earlier in 2002 compared to 2001.

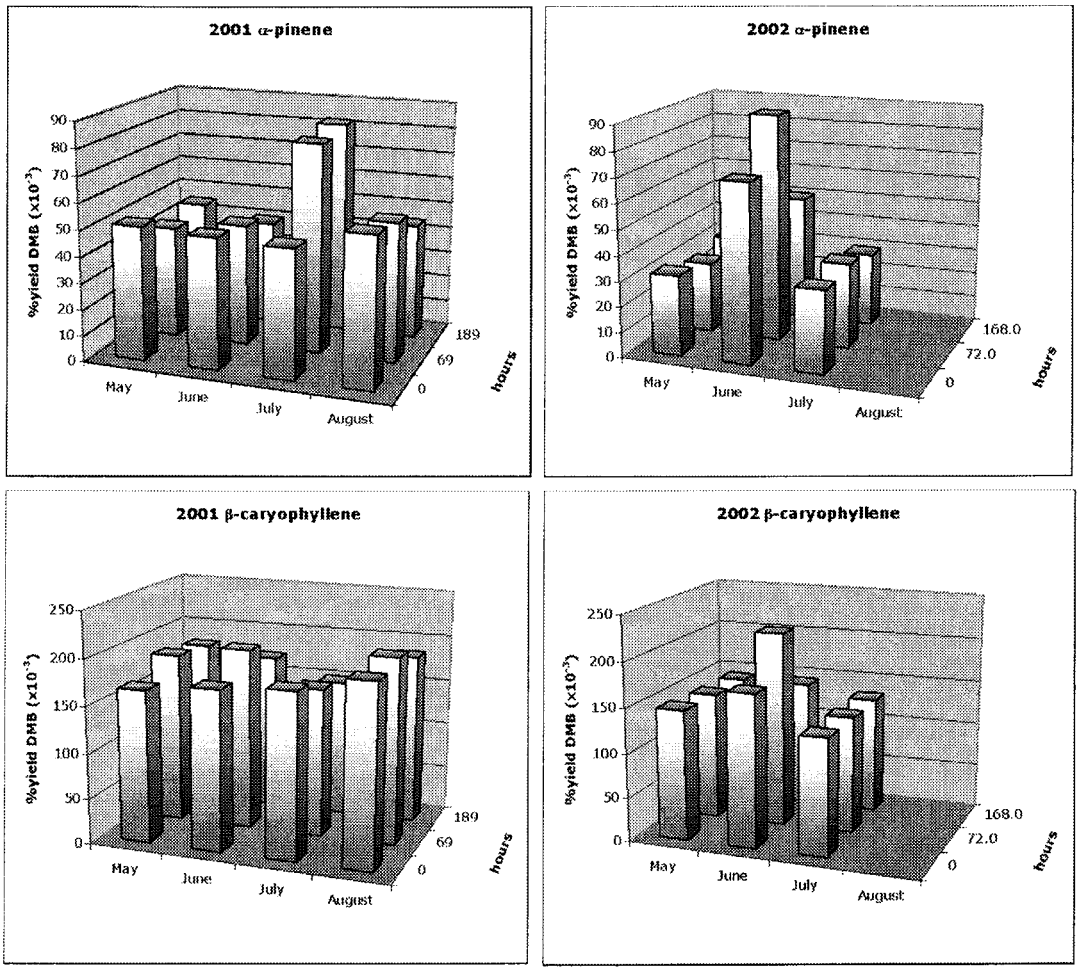


Figure 4.2.ii. Histograms of the %Yields of α-Pinene and β-Caryophyllene at the Relevant Harvest Dates and Following Incubation at 10°C in 2001 and 2002.

The increases effected by incubation in the lower molecular weight volatiles such as α-pinene, which exemplifies many of the monoterpenes, are also most evident in July in 2001 and June in 2002. Figure 4.2.ii shows the histograms of the harvest and

incubation results for α-pinene and β-caryophyllene in 2001 and 2002. Incubation was not as effective at increasing many sesquiterpenes as seen for β-caryophyllene in 2001 compared to the same experiment in 2002.

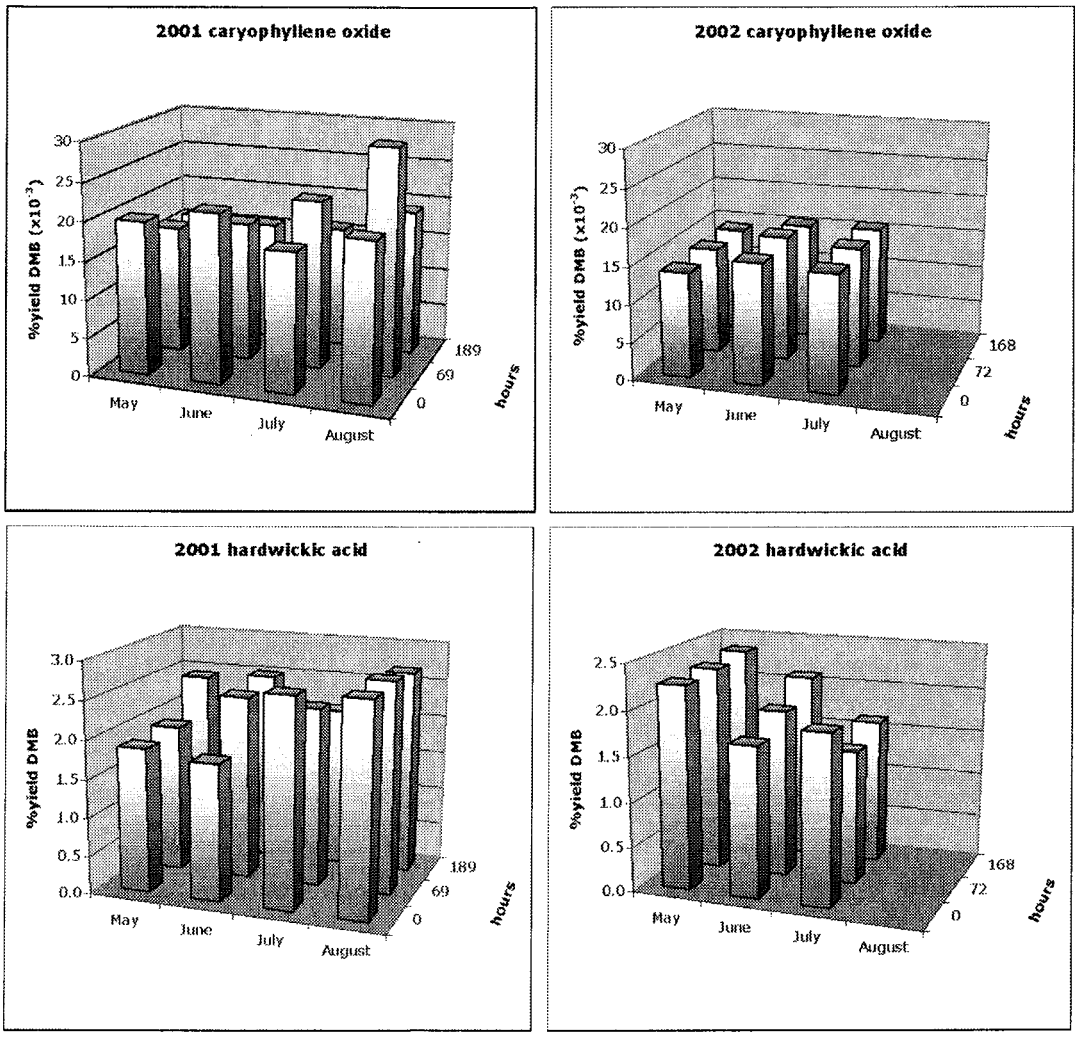


Figure 4.2.iii. Histograms of the %Yields of Caryophyllene Oxide and Hardwickic Acid at the Relevant Harvest Dates and Following Incubation at 10°C in 2001 and 2002.

Figure 4.2.iii shows the yields for caryophyllene oxide and hardwickic acid at the relevant harvest dates and incubation times. Post-harvest synthesis of oxygenated sesquiterpenes such as caryophyllene oxide is only effective for buds harvested in July

in 2001 and June in 2002 and was exhausted after 69 hours. High molecular weight components such as hardwickic acid trend towards an increase as bud burst approaches whilst post harvest incubation had limited effect. An increase in hardwickic acid as bud burst approaches was evident in the laboratory scale harvest trials conducted in the year 2000. The results presented in table 3.2.1.i (page107) show a 38 % increase in the 4 weeks preceding the initiation of bud burst at site 2 for White Bud buds and a 200 % and a 191 % increase at site 1 for White Bud and HTC buds respectivedly during the period preceding 23 % bud burst at site 2.

Predicting optimal harvest date then must be based on assessment of conditions for each season. In addition, industry does not have the option to select the date of harvesting due to the limitations of the harvesting machines which are required to continue operations from June through to August. Nonetheless incubation of buds after June does not appear to have detrimental effects on oil yield or volatile composition yet has the potential to increase both.

Balancing the integrated effect of harvest and incubation time to achieve a higher quality blackcurrant extract cannot solely rely on quantification of individual chemical components using analytical procedures such as gas chromatography. Assessment of the aromatic impact of the extracts produced is of fundamental importance.

Aroma Assessment of the harvest and incubation trials in 2001 & 2002

For both 2001 and 2002 the aroma assessments indicate that blackcurrant buds should not be harvested prior to May or June and that incubation of buds harvested in these months does not improve the sensory qualities of the extract. In the 2001 harvest year aroma assessments would indicate that harvesting in July and incubating buds for 72 hours would result in a product with the optimum cattiness. Incubation of buds in August also does not improve the cattiness based on aroma assessment. These observations did not correlate to the levels of 4-methoxy-2-methyl-2-butanethiol detected in the extracts that is thought to confer cattiness. The disparities between the degree of cattiness indicated by aroma assessment and the level of thiols ascertained by GC FPD analyses show that the aroma impact of extracts cannot be fully assessed using chemical analysis and quantification of individual components. Rather the

quality and impact of a product is a complex interaction. The only component whose concentration increased as the level of perceived cattiness increased as determined by the judges was α-thujene. This is based on a major component which is unlikely to explain the interactions reported here.

4.2.5. Commercial-scale Incubation of Blackcurrant Buds for Improved Extracts

The results for the commercial scale-incubation showed that incubation for 72 hours produced a 29 % increase in the level of 4-methoxy-2-methyl-2-butanethiol. This was offset by a drop in oil yield of 8 % over the 4 sets of concretes extracted. The introduction of incubation would therefore be best limited to accommodate markets that have specified a preference for oils with an increased level of cattiness.

Section 4.3. SYNTHESIS OF THIOLS

Using procedures described by Riguard *et al.*,(1986) the target product 2-chloro-4-methoxy-2-methyl butane was successfully synthesized as described in stage 1 of section 2.3.1. Recovery was low at 10.5 % however it was sufficient to proceed onto the second stage in the synthesis of 4-methoxy-2-methyl-2-butanethiol. Although the synthesis was repeated several times the target product was only present at a detectable level on one occasion. As such alternative methods of synthesis were devised.

The successful synthesis using methyl 3-methoxypropionate to produce the intermediate 4-methoxy-2-methyl-2-butanol allowed the thiolation of the product using Lawesson's reagent in toluene. Although the target product, 4-methoxy-2-methyl-2-butanethiol, could not be isolated from the toluene by fractional distillation, the chomatograms acquired using GC MSD indicated a good yield of the thiol, with very few contaminants evident. The excellent mass spectra obtained included the parent molecular ion at 134, with all fragment ions corresponding to those published (Riguard *et al.*, 1982). Although the blackcurrant extracts produced in Tasmania are naturally produced essential oils, ready access to the synthetic form of the component which confers the essential catty note would allow for future experimentation on determining threshold levels at which the chemical impacts on the aroma profile in local produce.

Section 4.4. SYNTHESIS OF THE CYSTEINE-THIOL CONJUGATE AND MONITORING OF THE THIOL PRECURSOR IN BLACKCURRANT BUDS AND EXTRACTS.

The release of odours reminiscent of blackcurrants in Sauvignon wines after fermentation prompted the first experiments into the release of thiols from non-volatile precursors in wine must (Darriet *et al.*, 1993). The successful identification of cysteine conjugated precursors in wines and the similarities in the molecular structures of thiols produced during fermentation and those detected in blackcurrant prompted the conjecture that the precursors were similar. The successful synthesis of the proposed conjugate (structure VIII, Materials & Methods 2.4) using 4-methoxy-2-methyl-2-butanol reacted with l-cysteine in trifluoroacetic acid facilitated the establishment of an analytical method using HPLC MS/MS.

The naturally occurring cysteine thiol conjugate was successfully detected when sodium metabisulphite, ascorbic acid and tartaric acid were used in the extraction process of blackcurrant buds. This is the first time that a cysteinylated thiol precursor has been identified in blackcurrant. The extraction and analytical procedures were used to establish that the levels of the cysteine-thiol conjugate in the HTC buds were higher than that detected in the standard White Bud variety. High thiol varieties had 3.3 fold the levels of endogenous thiol and 4.6 fold the level of the conjugate than those quantified in cv. White Bud. The correlation between the higher thiol and higher conjugate levels in the high thiol varieties further supports the proposition that the cysteine-thiol conjugate is indeed the precursor to this important odour active thiol in blackcurrants.

Researchers into viticulture have applied the analytical methods developed to quantify the aromatic potential of grapes and wine musts (Peyrot des Gachons *et al.*, 2000; Murat *et al.*, 2001). The methodology employed requires the introduction of eluant from flash chromatography to chelating columns with release of bound analytes using cysteine. Whilst flash chromatography was used in this study to establish the presence of cysteinylated thiol precursors in blackcurrant buds, a significant degree of variation in recoveries from identical bud samples precluded the extraction method for use in

quantitative studies. The development of a simple extraction method that excluded the need for flash chromatography or chelating columns may provide the essential oil industry in Tasmania with an effective tool to quantify the aromatic potential of blackcurrants.

The analytical procedures also allowed for the establishment of a time series trial to monitor the endogenous levels of the cysteine-thiol conjugate in conjunction with the volatile 4-methoxy-2-methyl-2-butanethiol during the final stages of dormancy up until bud burst. Although the absolute concentration of the conjugate could not be established it was shown that there was an overall inverse relationship in the levels of the 4-methoxy-2-methyl-2-butanethiol and the levels of the cysteine-thiol conjugate. If indeed the 4-methoxy-2-methyl-2-butanethiol had been produced from the cysteine-thiol conjugate then it would be expected that as the thiol levels increased the levels of the conjugate would decrease. Although both decreased in the first 14 days, an inverse relationship between the two analytes was established. As bud burst progressed in late August / early September the levels of thiol increased then stabilised as the buds developed into small leaves. Concurrently the increase in the levels of the precursor evidenced may be undertaken within the plant to provide a reserve for the activation of thiol production in response to possible external stimuli.

CONCLUSION

The research reported within this thesis has addressed many of the aspects of the conditions of harvest and post-harvest and of the procedures of extraction relevant to the essential oils industry. In addition the chemistry of the thiol endogenous to blackcurrants has been elucidated for the first time. The experiments undertaken dealt with a wide range of variables, each inter-related and affected by a limitless array of endogenous and exogenous processes. The pertinent fundamental conclusions drawn are as follows;

It is accepted that machine harvesting is the only viable method in terms of labour costs in the blackcurrant extracts industry. The retention of some of the quality characteristics associated with hand-harvesting, however may be achieved with the inclusion of a process of sieving to separate large amounts of extraneous material. This study has shown that a machine based on the traditional threshing devise displayed in plate 1 has the potential to return 92 % of extractable material with 85 % high yielding in terms of quality extract. The reduced volume of material would save 15 % of the solvent required for effective extraction whilst returning similar volumes of blackcurrant extract as that recovered from un-sieved buds. The installation of a sieving machine within the processing factory itself may make this alternative viable in terms of labour costs.

Application of the new extraction technology developed here within and based on the steeping of buds in ethanol prior to a partitioning of extractable components into non-polar organic solvents increased the yield of volatiles by 29 % whilst reducing the yield of acids by 61 %. The extracts produced in this manner were perceived to have a more pungent catty aroma reminiscent of the European product. High impact components such as α-pinene, were present at levels 10 times those recorded for the standard extraction method. Yields of β-mycrene, δ-3-carene and β-phellandrene, three of the components reported by Latrasse to be susceptible to oxidation, were all higher in the ethanol based extraction process as were the levels of the co-eluting β-phellandrene and limonene. The higher yield obtained in this study when ethanol was used provides a strong indication that enzymatic degradation is indeed retarded by the

addition of low molecular weight alcohols. Adaptations of this method to industry did not compromise extract quality however yields were reduced when adjustments such as the removal of solvents were introduced to accommodate the limitations of existing infrastructure within the Tasmanian essential oils industry.

Fundamental to the marketability of the Tasmanian product is the level of the thiol endogenous to blackcurrant buds. In extracts produced using established protocols this important component was found to dissipate rapidly with only 15 % detectable in samples stored for less than a month. The lability of 4-methoxy-2-methyl-2-butanethiol in blackcurrant extracts (section 3.1.11.i) was confirmed by the loss of 6 to 9 % after 24 hours with only 60 to 70 % remaining after 8 days. Additives such as propylene glycol and BHA which were included in the extracting solvent and in the final product did little to slow the rate of depletion. However, despite the lack of chemical evidence to the preservative properties of the propylene glycol and BHA, concretes retained a more potent blackcurrant aroma for over five years when compared to concrete produced without BHA.

The increase in the levels of the thiol in Tasmanian product has also been facilitated by the increase in plantations of the new selection of high thiol containing clones. The eluciation into the chemical profiles has identified that the new clones differ from the cv. White Bud variety of blackcurrants by containing higher levels of 4-methoxy-2-methyl-2-butanethiol, sabinene, myrcene, bicyclogermacrene and hardwickic acid in bud extracts. Conversely, cv. White Bud buds had significantly higher levels of β-caryophyllene, β-phellandrene and limonene. δ–3-Carene and terpinolene were not discriminating components. Throughout the period of this study the expansion of the areas dedicated to blackcurrant canes specifically for oil production has adopted this new material.

Maximising the levels of quality components through propagating selections of high yielding clones and by improved extraction techniques has been shown to be significant in improving yield and quality of blackcurrant extracts. This study has also elucidated the significance of harvest time with regard to the quality of the buds and the latent potential for post-harvest synthesis during in dormant buds through winter.

Bud weight and volatile concentrations decreased throughout dormancy, through at a rate that may be attributed to volatilisation and leaching. The decrease in bud weight and yield of components would suggest that the harvesting of buds at the end of the dormancy is not necessarily optimal. High molecular weight components, including diterpene acids increased as bud burst approached. However, aroma assessments indicated that blackcurrant buds should not be harvested prior to May or June despite the chemical profile indicating otherwise and that incubation of buds harvested in these months did not improve the sensory qualities of the extract. The consideration of harvest time must also be tempered by the growing conditions specific to each season.

Post-harvest technology was also identified as another area that may impact on yield and quality of blackcurrant extracts. The limitations of commercial operations necessitates that blackcurrant buds be frozen after harvest until sufficient quantities are accumulated for large-scale batch extractions. This study has shown that within 24 hours of freezing over 50 % of the naturally occurring thiol, 4-methoxy-2-methyl-2-thiol butane is lost as are 18 % of volatile components. Longer term freezing resulted in a steady overall loss of all volatile components. The detrimental aspects associated with this practice should not be assumed to be unavoidable. Controlled rates of freezing and the lowering of the freezing temperature to below -20°C are two of a number of options that require further investigation. Indeed it may be suggested that the results reported here within support the proposition that freezing may be avoided altogether. Storage at room temperature for 72 hours increased the levels of thiols in large-scale factory based extractions although extract yield was lower. Further study to compare the yield and quality of extracts stored at room temperature and extracted without freezing should be compared to those frozen prior to extraction. It is possible that the decrease in yield resulting from post-harvest incubation recorded in this study is offset by the retention of volatiles that would have been lost by the freezing event that is standard in commercial operations.

Not withstanding the option of omitting the freezing event altogether in commercial operations, the loss of quality volatile components during freezing may alternatively be offset by instigation of incubations after the buds are frozen. Although freezing

reduces the levels of volatiles it does not retard the potential for post-harvest synthesis in machine-harvested buds. Indeed this study proposes that the processes involved in post-harvest production of volatiles in un-frozen buds are different from those active in fresh buds and a degree of structural damage is required for continued activation of volatile production in thawed buds. Production in hand-cut buds was significantly retarded following a freezing event whilst machine-harvested buds benefited in terms of volatile production by being frozen prior to incubation. The break down of compartmentalisation and hence substrate availability may be the factor contributing to this phenomenon. More aggressive damage to bud structure by rolling, however, resulted in a dramatic depletion of volatiles and had a significantly greater detrimental effect in the retardation of post-harvest production than did freezing for lower molecular weight terpenes. Major structural trauma de-activated all processes leading to post-harvest synthesis of monoterpenes and sesquiterpenes and most likely contributed to the loss by volatilisation of these more volatile components. Rolling did not stop post-harvest synthesis of oxygenated sesquiterpenes and diterpene acids. The loss of post-harvest synthesis of these components by freezing indicated that the production evident in fresh material, may be due to temperature sensitive enzymatic pathways. Alternatively substrates released from the confines of cellular structures damaged by ice crystallisation may have extinguished non-enzymatic synthesis. The disparities in post-harvest production across the different terpenoid families are consistent with sub-cellular compartmentalisation and are an indication that the main intra-cellular site for biosynthesis of the higher molecular weight terpenoids may well be removed from that of monoterpenes.

The elucidation of chemical profiles during the dormancy and incubation of blackcurrant buds provided the impetus to apply incubation technology on a pilot-scale and on an industrial scale. The yield and quality of extracts were reliant on the month of harvest whilst incubation was only effective within two months of bud burst. The incubation of buds after June did not have detrimental effects on oil yield or volatile composition yet with correct monitoring of seasonal variations has the potential to increase both. The pilot-scale incubation of buds harvested in August did not improve the cattiness based on aroma assessment. However commercial scale-incubations

undertaken for 72 hours prior to freezing trialed on buds harvested close to bud burst in late July and in August produced a 29 % increase in the levels of 4-methoxy-2-methyl-2-butanethiol. This was offset by a drop in oil yield of 8 % over the four sets of concretes extracted. As previously discussed the possibility that the storage of buds at room temperature at the factory may facilitate post-harvest synthesis as well as provide an alternative to freezing buds whilst sufficient quantities are accumulated for large batch extractions. Thus increased yields and improved quality parameters are achieved by increased production whilst avoiding the loss incurred by freezing. The introduction of incubation has the potential to accommodate markets that have specified a preference for oils with an increased level of cattiness.

The component most identified with quality extracts, 4-methoxy-2-methyl-2-butanthiol has been a major focus of this study. As mentioned the introduction of machine harvesting was accompanied by a decrease in yield and quality. The loss of quality was identified in this study to be partly associated with the loss of the thiol. Cutting buds from the cane by hand minimised damage to the structure and the reduced exposure to oxidative conditions may account for higher thiol concentrations (4.6 $mgkg^{-1}$ DMB) detected in intact buds compared to those extracted from machine-harvested material (3.5 $mgkg^{-1}$ DMB, section 3.2.3). Post-harvest thiol production was most rapid in fresh hand-cut buds, a process retarded by mechanical harvesting. The detrimental effect of structural damage to post harvest thiol synthesis and accumulation in buds was further demonstrated by the reduction in thiol concentration recorded when machine-harvested commercial buds were further damaged by rolling. The application of fermentation and the introduction of cysteine-S-conjugate lyases into blackcurrant extracts would further elucidate the process involved in the bio-synthesis of thiols. Further experimentation to include enzyme inhibitors would provide further information into the processes involved in post-harvest thiol synthesis.

Indeed this study has progressed the research into the chemistry of the naturally occurring thiol and has provided the key to future research. The development of a synthetic method using methyl 3-methoxypropionate to produce the intermediate 4-methoxy-2-methyl-2-butanol with thiolation using Lawesson's reagent in toluene has

the potential to produce the chemical with high yields and at low cost. Although the blackcurrant extracts produced in Tasmania are naturally produced essential oils, ready access to the synthetic form of the component which confers the essential catty note would allow for future experimentation on determining threshold levels at which the chemical impacts on the aroma profile in local produce.

More importantly the naturally occurring cysteine thiol conjugate has been successfully detected when sodium metabisulphite, ascorbic acid and tartaric acid were used in the extraction process of blackcurrant buds. This is the first time that a cysteinylated thiol precursor has been identified in blackcurrant. The extraction and analytical procedures were used to establish that the levels of the cysteine-thiol conjugate in the HTC buds were higher than that detected in the standard White Bud variety. High thiol varieties had 3.3 fold levels of endogenous thiol and 4.6 fold the level of the conjugate that of White Bud. The correlation between the higher thiol and higher conjugate levels in the high thiol varieties further supports the proposition that the cysteine-thiol conjugate is indeed the precursor to this important odour active thiol in blackcurrants.

The development of a simple extraction method that excluded the need for flash chromatography or chelating columns may provide the essential oil industry in Tasmania with an effective tool to quantify the aromatic potential of blackcurrants. The successful application of sulphiting technology has implications for any research into cysteine-based chemistry in blackcurrants.

The analytical procedures provided for the establishment of a time series trial to monitor the endogenous levels of the cysteine-thiol conjugate in conjunction with the volatile 4-methoxy-2-methyl-2-butanethiol during the final stages of dormancy up until bud burst. There was an overall inverse relationship in the levels of the 4-methoxy-2-methyl-2-butanethiol and the levels of the cysteine-thiol conjugate.

In the context of improving the yield and quality of blackcurrant extracts this study has elucidated the effects of time and mode of harvest, the effects of bud damage and storage, the potential to release volatiles from non-volatile bud components and the

stability of quality components through freezing storage and in the final extract product. It has provided a substantial framework from which future studies can progress in terms of extraction technology, post-harvest synthesis and biosynthetic pathways to the endogenous thiol.

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APPENDIX A

component	p-value			
2001	block effect	effect of incubation hours	effect of month	Interaction of incubation time x month
oil yield	0.0997	<0.0001	<0.0001	<0.0001
yield of volatiles	0.0542	0.0015	<0.0001	<0.0001
4-methoxy-2-methyl-2-butanethiol	<0.0001	<0.0001	<0.0001	<0.0001
thujene	0.0002	0.0178	<0.0001	<0.0001
α-pinene	0.0157	0.3516	<0.0001	<0.0001
sabinene	0.1962	<0.0001	<0.0001	<0.0001
mycrene	0.0593	<0.0001	<0.0001	<0.0001
δ-3-carene	0.4910	0.0025	<0.0001	<0.0001
α-phellandrene+limonene	0.0051	0.0274	<0.0001	<0.0001
ocimene	0.0106	0.0155	<0.0001	<0.0001
unknown 1	0.0054	0.0387	<0.0001	<0.0001
α-terpinolene	0.0233	0.0421	<0.0001	<0.0001
β-caryophyllene	0.0040	0.0503	0.0010	0.1378
humulene	0.0065	0.0303	<0.0001	0.0423
germacrene D	0.6312	0.0766	<0.0001	0.0981
bicyclogermacrene	0.6795	0.1541	<0.0001	0.0030
caryphylene oxide	0.0301	<0.0001	0.0530	0.0014
unknown 2	0.0432	<0.0001	<0.0001	<0.0001
unknown 3	0.4356	<0.0001	<0.0001	0.0041
polyanthic acid	0.0224	0.0264	0.0001	0.0004
hardwickic acid	0.1953	0.2442	<0.0001	<0.0001
oxo-hardwickic acid	0.0261	0.3273	0.0003	0.0036
hardwickic hydroxy acid	0.2092	0.7274	0.0049	0.0014

Table A1. The P-values for Dependent Variables of the Effect of Block, Incubation Hours, Month and the Inter-reaction Between Month and Incubation Hours for the Year 2001.

component	p-value			
2002	block effect	effect of incubation hours	effect of month	Interaction of incubation time x month
oil yield	0.0293	0.8953	0.0002	0.8661
yield of volatiles	0.0181	0.1009	<0.0001	0.0492
4-methoxy-2-methyl-2-butanethiol	0.0240	0.0533	0.0010	<0.0001
thujene	0.4405	0.0678	0.0014	0.1679
α-pinene	0.0989	0.4870	<0.0001	0.0207
sabinene	0.0029	0.0763	<0.0001	0.0894
mycrene	0.0099	0.0233	<0.0001	0.0204
δ-3-carene	0.0481	0.1112	<0.0001	0.0889
α-phellandrene+limonene	0.1002	0.1865	<0.0001	0.2041
ocimene	0.0685	0.0235	<0.0001	0.0083
unknown 1	0.0530	0.0481	<0.0001	0.0317
terpinolene	0.0335	0.1125	<0.0001	0.1240
β-caryophyllene	0.0040	0.1802	0.0070	0.1650
humulene	0.0007	0.3057	0.0328	0.4011
germacrene D	0.0018	0.2635	0.0197	0.3375
bicyclogermacrene	0.0011	0.1404	0.0012	0.1433
caryphyllene oxide	0.0072	0.8640	0.0268	0.9833
unknown 2	<0.0001	0.0002	<0.0001	0.2005
unknown 3	0.0011	0.0037	<0.0001	0.0588
polyanthic acid	0.0072	0.7950	0.0041	0.6860
hardwickic acid	0.0033	0.7219	0.0040	0.5452
oxo-hardwickic acid	0.0581	0.2423	0.0466	0.4813
hydroxyhardwickic acid	0.5084	0.2422	0.0046	0.1467

Table A2. The P-values for Dependent Variables of the Effect of Block, Incubation Hours, Month and the Inter-reaction Between Month and Incubation Hours for the Year 2002.

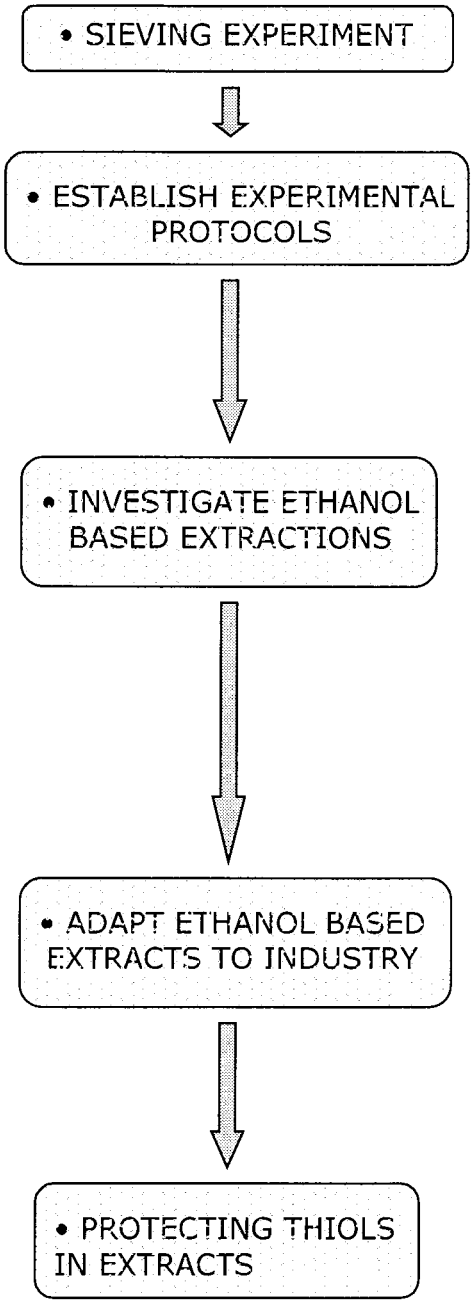
APPENDIX B

HARVEST AND EXTRACTION

primary focus

improving quality through;

* extraction process
* solvents
* additives



- potential to improve extract quality whilst lowering extraction volumes

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- masceration
- effective solvent ratios
- relating yield of solvated extracts to actual extract yields

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- trial ethanol based extracts back extracted with hexane/petroleum ether
- should buds be removed before buds are back extracted?
- investigate the effectiveness of solvents such as ethyl acetate and propylene glycol
- can volatile components be protected through extraction?
- could storage of buds in ethanol, as the first step in new extraction, reduce loss of thiols?

- can ethanol volumes be reduced as expensive and unmanageable?
- determine minimum ethanol volumes for effective extraction
- can the reduction of volume for ethanol by RVE be omitted as difficult?
- can the addition of water be omitted to allow solvent recycling?

- do thiol levels deplete and if so at what rate?
- can the depletion be slowed by the use of antioxidants, humeticants and solvents?

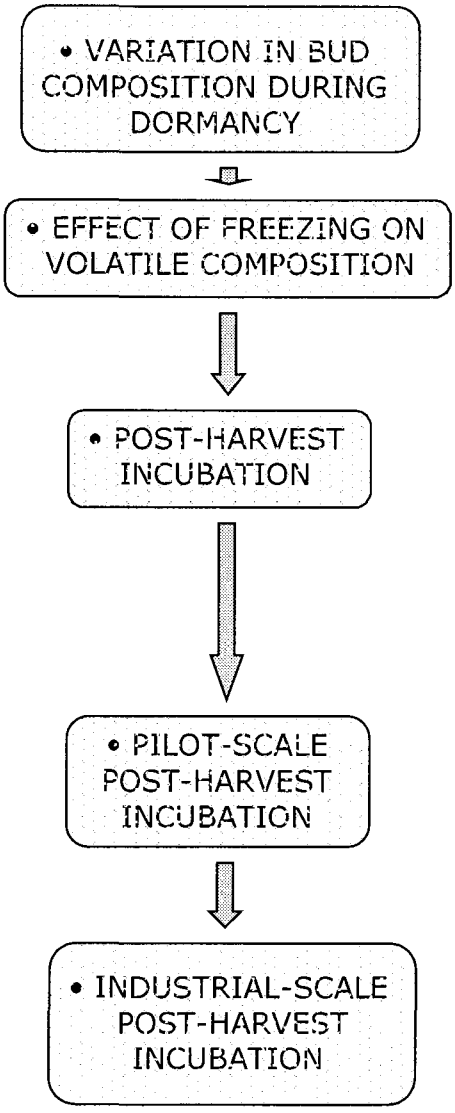
DORMANCY FREEZING & INCUBATION

primary focus

optimal harvest time

effect of freezing on volatile concentration

post-harvest volatile synthesis



- field trials conducted from late summer to late winter up until bud burst
- data process to determine optimal time to harvest?

- establish time series trials with frozen buds

- can volatile production continue after bud harvest?
- if production continues establish time series to determine duration of post-harvest synthesis
- does damage to bud structure affect post-harvest synthesis?
- does freezing de-activate post-harvest synthesis?

- determine optimal time of bud harvest for yield and quality of extract
- determine optimal time of bud harvest for post-harvest incubation
- assess the impact of both parameters on extract aroma in addition to component profile

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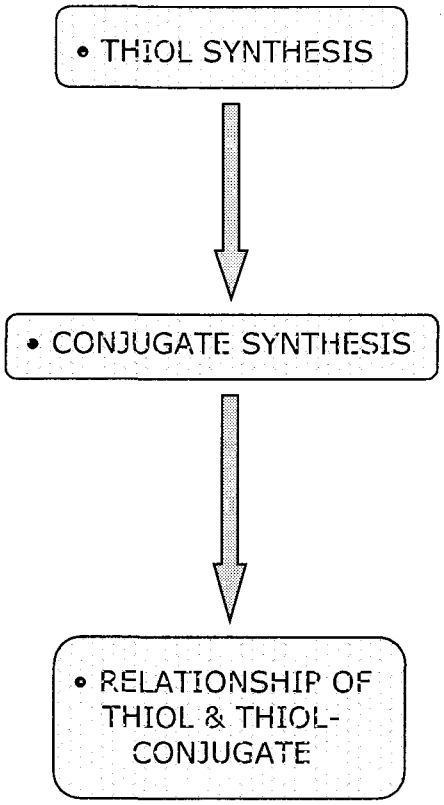
- can the results from the preceding trials be repeated on an industrial scale?

SYNTHESIS OF THIOL AND PRECURSOR TECHNOLOGY

primary focus

synthesis of 4-methoxy-2-methyl-2-butanethiol
identify precursor to thoil
establish relationship between precursor and thiol
determine levels in dormant buds



- synthesis of thiol using established synthetic protocols
- investigate alternate synthetic methods

- investigate likely precursor based on similar chemicals found in published literature
- synthesis of possible precurosr
- establish extraction protocol
- identify conjugate in extracts of blackcurrant bud
- validate repeatability of extraction with view to preliminary analytical methodology to be used in field trials

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- do high levels of thiol correspond to high levels of thiol-conjugate in buds?
- does the direct relationship translate across clonal stock?
- what is the relationship between the thiol and the thiol conjugate over time as bud burst approaches?

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Garland, S. M., Menary, R. C., Claye, C. J., 2002, Variation during dormancy and the effect of freezing and postharvest incubation on the chemical composition of blackcurrant buds (Ribes nigrum L.), Journal of horticultural science and biotechnology, 77(4), 489-497